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The impact of inflammation on muscarinic receptor subtypes expression

Immunohistochemical examination of lacrimal and salivary glands of rat and human

Vliv zánětu na expresi podtypů muskarinových receptorů

Imunohistochemie slzních a slinných žláz potkana a člověka

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Hradec Králové, 2010

I declare that my diploma thesis is my original work. All used literature and other sources are named in References and quoted properly.

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

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Contents

Abstract

This project was focused on the characterization of muscarinic receptor subtype expression in lacrimal and salivary glands of the rat. Immunohistochemical methods were used to reveal the presence of the M1, M3 and M5 receptors and the expression of these subtypes was further studied under experimentally induced inflammatory conditions. The expression of the above mentioned muscarinic receptor subtypes was also studied in human labial glands, both healthy and with autoimmune sialadenitis compatible with Sjögren's syndrome.

M1, M3 and M5 muscarinic receptors were found in the lacrimal and submandibular glands of the rat using immunoenzyme and immunofluorescent methods. The M5 receptor in the lacrimal gland was also detected on intracellular, possibly nuclear localization. The tissue with induced inflammation didn't show any significant differences in the muscarinic receptor expression. The immunofluorescent labeling of the M1 receptor in human labial glands with Sjögren's syndrome showed slight down-regulation of this subtype. The M3 receptor was up-regulated, as well as the M5 receptor, which showed apparent up-regulation.

Abstrakt

Tento projekt byl zaměřený na charakterizaci exprese podtypů muskarinových receptorů v slzních a slinných žlázách potkana. K odhalení přítomnosti M1, M3 a M5 receptorů byly využity imunohistochemické metody a exprese těchto podtypů byla dále studována při experimentálně navozeném zánětu. Exprese výše zmiňovaných podtypů muskarinových receptorů byla taktéž studována v lidských pyskových žlázách zdravých a postižených autoimunitní sialadenitídou kompatibilní se Sjögrenovým syndromem.

Muskarinové receptory M1, M3 a M5 byly nalezeny v slzních a podčelistních žlázách potkana s využitím imunoenzymové a imunofluorescenční metody. M5 receptor byl v slzní žláze detekován v nitrobuněčném, eventuálně jaderném umístění. Tkáň s navozeným zánětem nevykazovala žádné významné změny v expresi muskarinových receptorů. Imunofluorescenční značení M1 receptoru v lidských pyskových žlázách se Sjögrenovým syndromem naznačilo mírnou down-regulaci tohoto podtypu. M3 receptor byl up-regulovaný, společně s M5 receptorem, který vykazoval výraznou up-regulaci.

1 Introduction

The autonomic nervous system regulates various functions throughout the body. This includes secretory as well as contractile functions, e.g. glands in the gastrointestinal tract and smooth muscles in the circulatory system. These effects are generally very well described in the literature.

However, besides the classical effects of the autonomic nervous system, it participates in the cross-talk with the immune system and has recently been described to affect inflammation, proliferation and tumor development.

The impact of changes of the parasympathetic influence on lacrimal and salivary glands has been thoroughly studied in the chronic autoimmune inflammatory disease Sjögrens's syndrome, which is primarily manifested by xerostomia and xerophtalmia. These conditions may seriously affect the quality of life of the patient and may also lead to further complications. This syndrome and inflammation in general may lead to alteration of the muscarinic receptor subtype expression pattern. This was shown both in Sjögren's syndrome labial glands, as well as in the urinary bladder during cystitis conditions. These changes in muscarinic receptor expression have been shown to be of functional significance in state of inflammation.

The expression of muscarinic receptors is well studied in salivary glands under normal conditions. However, in the lacrimal gland, there are only few reports, which leave space for further elucidation.

2 Review of literature

2.1 The autonomic nervous system

The autonomic nervous system regulates various functions all over the body on the unconscious level and its main aim is to ensure self-preservation by maintaining homeostasis in the continuously changing environment. The ANS provides innervation to all organs of the body, except for the skeletal muscle, which is supplied by the somatomotor nervous system. The main functions regulated include the heart frequency, contraction and relaxation of smooth muscle, all exocrine and certain endocrine secretions and energy metabolism such as glucose utilization (especially in liver and skeletal muscle). (Rang 2003)

We can distinguish two major divisions of the ANS: the sympathetic and the parasympathetic nervous systems. Additionally, the ANS includes the enteric nervous system within the gastrointestinal tract. (Barrett and Ganong 2010) The latter one is capable of functioning independently of the central nervous system, in contrast to the sympathetic and parasympathetic divisions. In the classical view, the sympathetic and parasympathetic systems were apprehended as mere antagonists, but this has been shown to be true only in some situations, such as the control of heart rate or the gastrointestinal smooth muscle, while in e.g. salivary glands, they have complementing functions. (Rang 2003)

The main difference between the autonomic and somatic efferent pathways is that the autonomic pathway consists of two neurons, preganglionic and postganglionic, arranged in series, while the somatic pathway consists of a single motoneuron connecting the CNS to the skeletal muscle fibre. This means that all the actual efferent neurons lie outside the CNS, grouped to form ganglia. The preganglionic fibres passing from CNS to the ganglia are slow-conducting class B and C and release acetylcholine from their nerve terminals. The postganglionic fibres running from the ganglia to the tissues are slow-conducting, unmyelinated class C fibres and they travel together with fibres from the visceral sensory receptors forming the visceral nerves. (Petersen 2007)

Until the late 1970s, chemical transmission from autonomic nerves to their peripheral effector tissues generally had been thought to be mediated exclusively by norepinephrine or acetylcholine. However, even before the transmitter concept was

established, Heidenhain noticed that the muscarinic antagonist atropine did not eliminate all the functional responses to parasympathetic nerve stimulation of salivary glands. Later on, Johansson and Lundberg showed that acetylcholine and vasoactive intestinal peptide (VIP) are co-localized in certain nerve endings in the submandibular salivary gland and lacrimal gland. (Johansson and Lundberg 1981) Another very significant observation was that acetylcholine caused relaxation of rabbit artery in the presence, but not in the absence of endothelium, thus cholinergic relaxation was shown as completely dependent on an endothelium-derived relaxing factor, which was in due course identified as nitric oxide. (Furchgott 1993) A subsequent body of evidence led to the general acknowledgement of the non-adrenergic, non-cholinergic transmission within the ANS. These transmitters include peptides, e.g. neuropeptide Y, substance P and VIP, the gas nitric oxide (NO) and adenosine-5'-triphosphate (ATP). (Lundberg 1996)

2.1.1 The sympathetic nervous system

The sympathetic division of the ANS is sometimes also referred to as the thoracolumbar portion, because the preganglionic cell bodies are situated in the thoracic and upper lumbar segments of the spinal cord (T1-L3). Their myelinated fibres exit the spinal cord via the anterior spinal roots and then travel through the white rami communicantes to the paravertebral chain of sympathetic ganglia situated bilatteraly next to the spinal cord. The cell bodies of the postganglionic neurons are located here and their axons rejoin the spinal nerves through the grey rami communicantes for distribution to sweat glands and pilomotor muscles and to blood vessels of skeletal muscle and skin. However, the sympathetic fibres that innervate abdominal and pelvic viscera don't travel through the spinal nerves; they leave the paravertebral ganglia in visceral nerves instead. The postganglionic cell bodies are to be found in the unpaired prevertebral ganglia in the abdominen and the pelvis. The third type of sympathetic ganglia are the terminal ganglia located near the organs that they innervate; they include the ganglia connected to the urinary bladder and rectum and the cervical ganglia in the region of the neck. (Goodman, Hardman et al. 2001)

An interesting exception of the two-neuronal configuration is the innervation of the adrenal medulla, where the preganglionic sympathetic fibres don't synapse in the

ganglia, but travel directly to their destination, where they release acetylcholine. The cells of the adrenal medulla can thus be considered as modified postganglionic sympathetic neuronal cells, releasing epinephrine and norepinephrine into the blood stream. (Petersen 2007)

The sympathetic nervous system uses acetylcholine and norepinephrine as transmitters. Acetylcholine is released by preganglionic fibres of both sympathetic and parasympathetic fibres, and acts on nicotinic receptors. Norepinephrine, which acts on either α or β receptors, is released by all the postganglionic fibres, with the exception of the sweat glands, where transmission is mediated by acetylcholine acting on muscarinic receptors. However, cotransmission is a general phenomenon in the ANS and non-adrenergic, non-cholinergic transmitters also play a significant role in the autonomic transmission. The main NANC transmitters of the sympathetic system are ATP and neureptide Y. (Rang 2003)

Activation of the sympathetic nervous system is primarily aimed at coping with stress, known as the "fight or flight" response. During rage and fright, all the sympathetically innervated structures of the body are affected simultaneously. This results in increased heart rate and contractility, pupillary dilatation, bronchodilatation, inhibition of gastrointestinal motility, constriction of the splanchnic vessels, elevation of glycaemia and free fatty acids, etc. Many of these effects are primarily casued by the release of epinephrine from the adrenal medulla. In addition, signals are conveyed to higher brain centers to facilitate meaningful responses or to imprint the event in memory. However, most of the activity of the sympathetic system is linked to homeostatic activity and it exerts localized actions, while its activity varies from organ to organ. (Goodman, Hardman et al. 2001) A more detailed description of the effects of sympathetic activation is given in Table 1.

2.1.2 The parasympathetic nervous system

The preganglionic cell bodies of the craniosacral division of the ANS are situated in the sacral spinal cord (S2-4), the midbrain and the brainstem; the fibres from the brain are included in the III. (oculomotor), VII. (facial), IX. (glossopharyngeal) and X. (vagus) cranial nerves. The ganglia lie very close to the affected organs, resulting in the fact that the postganglionic nerves are very short in comparison with the sympathetic.

The neurons from the sacral portion travel to the pelvic ganglia, which carry both sympathetic and parasympathetic fibres.

The major transmitter of the parasympathetic nervous system is acetylcholine, released by preganglionic, as well as postganglionic neurons. Acetylcholine acts on nicotinic and muscarinic receptors. NANC transmission also plays an important role, mainly via VIP and nitric oxide. (Rang 2003)

The parasympathetic division is primarily aimed towards maintenance and conservation of bodily function. The main responses upon stimulation are decreased heart rate, contraction of the urinary bladder, constriction of the pupils and the ciliary muscle and increased motility and secretion in the gastrointestinal tract. (Petersen 2007)

2.1.3 The enteric nervous system

The third portion of the ANS is very different from the previous two, as it forms a broad network where many cells are not affected by the CNS. The whole network of ganglia and their connecting axons is situated in the wall of the intestines, creating plexuses. The myenteric (Auerbach's) plexus is involved in control of digestive tract motility, while the submucosal (Meissner's) plexus regulates gastrointestinal blood flow and epithelial cell function. (Barrett and Ganong 2010) The enteric system contains numerous neurons transformed into mechano- and chemoreceptors which sense the environment of the intestinal lumen and can provide local reflex pathways without external input. The enteric nervous system is pharmacologically very complex and it involves various transmitters, such as neuropeptides, serotonin, NO and ATP, as well as acetylcholine and norepinephrine. (Rang 2003)

Table 1. *The major effects of the autonomic nervous system*

Organ	**Sympathetic effect**	**Adrenergic receptor type**	**Parasympathetic effect**	**Main cholinergic receptor type**
Heart				
Sinoatrial node	↑ rate	β_1	↓ rate	M_2
Atrial muscle	↑ force	β_1	↓ force	M_2
Atrioventricular node	↑ automaticity	β_1	↓ conduction velocity Atrioventricular block	M_2
Ventricular muscle	↑ automaticity ↑ force	β_1	No effect	M_2
Blood vessels				
Arterioles				
coronary	constriction	α		
muscle	dilatation	B_2	No effect	
viscera	constriction	α	No effect	
skin	constriction	α	No effect	
brain	constriction	α	No effect	
erectile tissue	constriction	α	Dilatation	? M_3
salivary gland	constriction	α	Dilatation	? M_3
Veins	Constriction	α	No effect	
	Dilatation	β_2	No effect	
Viscera				
Bronchi smooth muscle	No sympathetic innervation, but dilated by circulated	β_2	Constriction	M_3

	adrenaline			
Glands	No effect		Secretion	M_3
Gastrointestinal tract				
Smooth muscle	↓ motility	$\alpha_1, \alpha_2, \beta_2$	↑ motility	M_3
Sphincters	Constriction	α_2, β_2	Dilatation	M_3
Glands	No effect		Secretion	M_3
			Gastric acid secretion	M_1
Uterus				
pregnant	Contraction	α	Variable	
non-pregnant	Relaxation	β_2		
Lacrimal glands	?		Secretion	M_3
Salivary glands	Secretion	α, β	Secretion	M_3 M_1
Eye				
Pupil	Dilatation	α	Constriction	M_3
Ciliary muscle	Relaxation (slight)	β	Contraction	M_3
Kidney	Renin secretion	β_2	No effect	
Skin				
Sweat glands	Secretion (mainly cholinergic)	α	No effect	
Pilomotor	Piloerection	α	No effect	
Liver	Glycogenolysis Gluconeogenesis	α, β_2	No effect	
Male sex organs	Ejaculation	α	Erection	? M_3

Adapted from: *Rang, H. P. (2003). Pharmacology. Edinburgh; New York, Churchill Livingstone.*

2.2 Muscarinic acetylcholine receptors

Muscarinic receptors are responsible for the responses to parasympathetic stimulation mediated by the release of acetylcholine by parasympathetic postganglionic nerve fibres on the effector cells. Currently, five distinct muscarinic receptor subtypes are known, the M1, M2, M3, M4 and M5 receptors. They regulate numerous basic physiological functions and may have different roles within the same body system, with potential interplay between the subtypes, and they also play various roles in both central and autonomic nervous systems.

Their primary location in the peripheral nervous system is on autonomic effector cells innervated by postganglionic parasympathetic fibres. They can also be found in the ganglia and tissues with little or no cholinergic innervation, for instance on the endothelial cells of blood vessels (Tracey and Peach 1992) and are also present in high density in some structures of the central nervous system, where they are involved in various functions, such as motor control, temperature regulation, cardiovascular regulation and memory (Caulfield and Birdsall 1998). They are structurally much different from the other acetylcholine-activated receptors located in the autonomic ganglia and neuromuscular junctions, the nicotinic receptors. The nicotinic receptors consist of five protein subunits and are coupled to an ion-channel, while the muscarinic receptors are composed of only one protein, which has the same general structural features common to all members of the superfamily of seven transmembrane-helix G-protein coupled receptors. (Felder 1995)

The classical approach to receptor classification is based on pharmacological agonists and antagonists. At first, two subtypes, the M1 and M2 receptors were distinguished on the basis of selective affinity to pirenzepine. (Hammer, Berrie et al. 1980) Later on, four distinct muscarinic receptor genes lacking introns in the coding sequence subtypes were identified through the method of molecular cloning by Bonner (1987) and this was followed by the cloning of the gene for the fifth muscarinic acetylcholine receptor. (Bonner, Buckley et al. 1987; Bonner, Young et al. 1988) The gene sequences show significant homologies with the other members of the G-protein-coupled receptor superfamily (Hulme, Birdsall et al. 1990) and there is also a high level of similarity among different mammalian species.

Structurally, the muscarinic receptors consist of seven hydrophobic transmembrane helices linked together by three intracellular and three extracellular loops. The

intracellular third loop is critically involved in the process of determination of coupling selectivity and triggering different G-protein activation of M1/M3/M5 and M2/M4 subtypes. (Wess, Liu et al. 1997) The receptor protein has an intracellular carboxy terminus and and extracellular amino terminus.

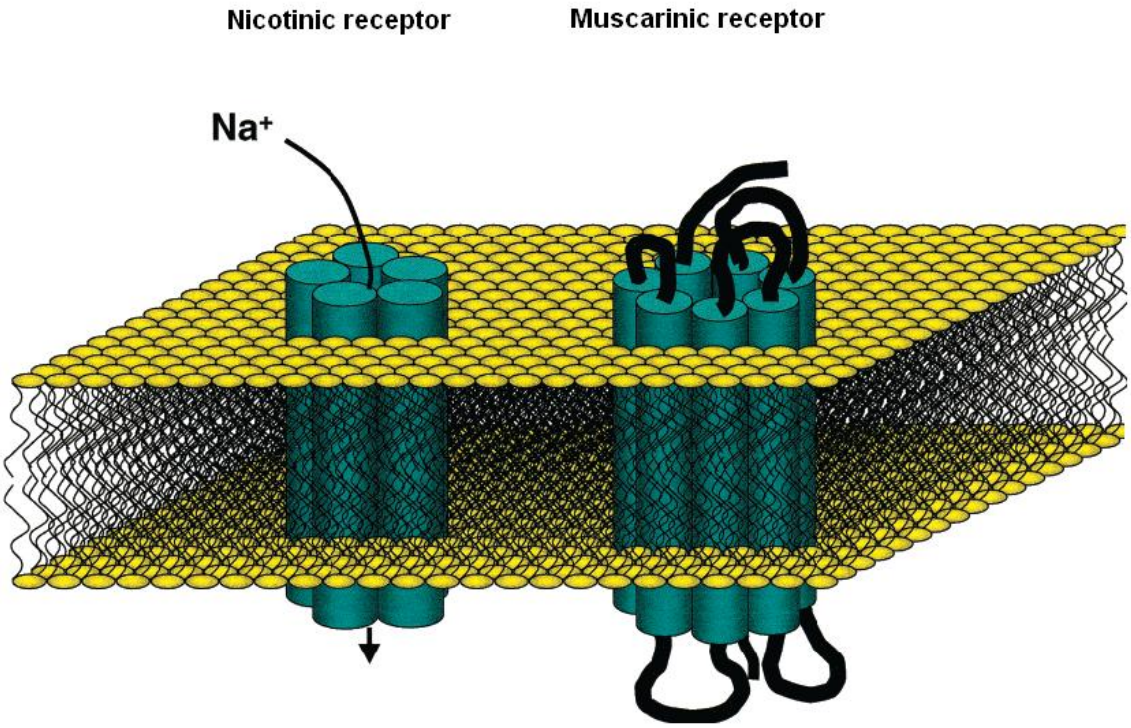

Fig. 1. Schematic drawing of structure of the nicotinic and muscarinic receptor
*Adapted from: Felder, C. C., F. P. Bymaster, et al. (2000). "Therapeutic opportunities for muscarinic receptors in the central nervous system." J Med Chem **43**(23): 4333-53.*

2.2.1 G-protein signalling

G proteins form a family of evolutionarily conserved, plasma membrane-associated signal transducers composed of an α subunit, which binds the guanine nucleotides GDP or GTP, and a tightly associated βγ heterodimer subunit. In mammals there are 20 α, 5 β, and 11 γ chains that combine to form many distinct G-protein subtypes. The α subunits, which are used to name the G protein, are divided by structure into four main subfamilies (αi/o, αq, αs, and α12/13). (Paul 2003) They contain two domains: a GTPase domain that is involved in the binding and hydrolysis of GTP and a helical domain that buries the GTP within the core of the protein. The helical domain is the

most divergent domain among Gα families and may play a role in directing specificity of receptor- and effector- G protein coupling. (Cabrera-Vera, Vanhauwe et al. 2003)

Bacterial exotoxins have been proved as valuable tools in the identification of G proteins. Cholera toxin activates Gs by catalyzing ADP-ribosylation of specific amino-acid residues in the α subunit, and therefore disables the hydrolysis of its bound GTP. Pertussis toxin blocks the receptor-mediated activation of Gi and Go, also by ADP-ribosylation of the α subunit, resulting in the complex remaining bound to GDP. (Petersen 2007)

In the classic G-protein activation cycle, the binding of an agonist converts the receptor into a GEF (guanine nucleotide exchange factor), which catalyzes the exchange of GDP for GTP on the α subunit. This causes the G protein to leave the receptor, to dissociate into α and βγ subunits, which remain tethered to the plasma membrane by covalently attached lipid anchors on the α and γ chains, and to activate downstream intracellular effectors. The α subunit possesses slow intrinsic GTPase activity, and therefore acts as a timer. The intrinsic GTPase activity can be accelerated by one or more RGS proteins (regulators of G-protein signaling), these are the GTPase activating proteins for heterotrimeric G proteins. The result of this net is restoration of the GDP-bound form of the α subunit, which then reassociates with βγ subunits and returns to new unoccupied receptors, thus completing the cycle. (Paul 2003)

2.2.2 Muscarinic receptor subtypes

Muscarinic M1, M3 and M5 receptors are sometimes also called excitatory receptors, and are located mostly postsynaptically. They couple to pertussis toxin-insensitive $G_{q/11}$, which is responsible for the activation of phospholipase C (PLC) that breaks down a minor membrane phospholipid, phosphatidylinositol bisphosphate (PIP_2) into 1,4,5-inositol triphosphate (1,4,5-IP_3) and diacylglycerol (DAG). 1,4,5-IP_3 binds to and activates its receptors located in intracellular Ca^{2+} stores, e.g. the endoplasmatic reticulum. This interaction results in the release of Ca^{2+} into the cytosol. Activation of M_1, M_3, and M_5 receptors can also trigger the activation of phospholipase A_2, leading to the release of arachidonic acid and consecutive eicosanoid synthesis. (Felder 1995)

M2 and M4 receptors are presynaptic and preferentially couple to pertussis-toxin sensitive Gαi/o, which inhibits the activity of the enzyme adenylate cyclase and thereby

lowers the level of cAMP. In M2 signalling, the βγ subunit also directly activates the inward rectifier K^+ channels in the heart. (Wickman and Clapham 1995)

Most cells, tissues and organs express multiple muscarinic acetylcholine receptor subtypes and while in some tissues one subtype may predominate in mediating a particular functional response, in others they may be of equal significance.

The M1 receptors are widely distributed in several structures of the brain, such as the hippocampus, neocortex and neostriatum. They are tightly connected to the processes of memory and learning, so agonists for this subtype have been proposed as potential treament for dementia or decrease of cognitive functions associated with Alzheimer's disease, aging or schizophrenia. They are expressed in some parts of the eye (Gil, Krauss et al. 1997), namely in the iris, sclera and lens epithelial cells, but their role hasn't been completely elucidated yet. This subtype plays a role in salivation, as discussed further, and is significant mainly for the the control of high-viscosity lubrication. In the nerve terminals of the urinary bladder, the prejunctionally located M1 receptors facilitate transmitter release. (Chess-Williams 2002) In non-neuronal cholinergic functions, the M1 receptor has a role in immune response, as M1- knockout mice suffered from a defect in cytolytic T lymphocyte differentiation. (Zimring, Kapp et al. 2005) A role for the M1, along with M2 and M3 subtype, has also been proposed in tumor angiogenesis. (de la Torre, Davel et al. 2005)

The M2 receptor is abundant both in central and peripheral nervous systems. (Oki, Takagi et al. 2005) Selective M2 antagonists increase cholinergic overflow, as they reduce autoreceptor inhibitory function in both brain and the periphery, so they can be approached in the therapy of Alzheimer's disease. (Eglen 2006) The M2 subtype has a prominent function in the heart, where it modulates pacemaker activity, atrioventricular conduction and force of contraction. (Dhein, van Koppen et al. 2001) The bradycardia-inducing effect was proved also by experiments on M2 knockout mice, where M2-agonist- induced bradycardia was eliminated. (Stengel, Gomeza et al. 2000) In the brain, M2 receptors are expressed in the caudate putamen, where they act as inihibitory heteroreceptors on dopaminergic terminals (Eglen 2006), and they were also suggested to take part in cognitive processes (Tzavara, Bymaster et al. 2003). In the detrusor muscle of the urinary bladder, the M2 receptors are even more densely expressed than the M3 receptors, even though their function is not completely clear. Experiments on knockout mice suggest that they may take part in the indirect mediation of bladder contractions by enhancing the contractile response to M3 receptor activation, and that

they may also mediate minor contractions to a certain extent. (Ehlert, Griffin et al. 2005) The M2 receptors also predominate in the smooth muscles of lung, stomach, ileum and colon. (Levey 1993) In the lung, they are involved in the parasympathetic bronchial control and it seems according to experiments on murine airways that constriction is mediated by accordant activation of M2 and M3 receptors. (Struckmann, Schwering et al. 2003) In the gastrointestinal tract, they again outnumber the M3 receptors (Gomez, Martos et al. 1992), but here they contribute to the contractility more than in the bladder. (Matsui, Motomura et al. 2000)

M3 receptors are also to be found in the central nervous system, but to lesser extent than the other subtypes. (Felder, Bymaster et al. 2000) Their central roles have not been completely elucidated yet, but the results of Yamada suggest that they may be involved in regulation of food intake. (Yamada, Miyakawa et al. 2001) This subtype mediates contraction of many types of smooth muscle in the respiratory, gastrointestinal and genitourinary organ systems. It plays a significant role in stimulation of gastrointestinal motility. (Matsui, Motomura et al. 2002) In the bladder, it predominantly mediates contraction of the detrusor muscle. The M3 receptors are also present in exocrine glands, namely the lacrimal and salivary glands, where they are the main subtype responsible for secretion. The M3 subtype is dominant also in the structures of the eye and it predominantly mediates ciliary muscle contraction, resulting in the classic effect of cholinergic agonists, miosis. (Poyer, Gabelt et al. 1994) In non-neuronal cholinergic function, the M3 receptor has been shown to play a role in vascular relaxation via NO release (Eglen and Whiting 1990), tumour growth and proliferation (Song, Sekhon et al. 2003), inhibition of cell migration (Chernyavsky, Arredondo et al. 2004) and inhibition of apoptosis (Budd, Spragg et al. 2004).

In the central nervous system, the M4 receptors are co-localized with the dopaminergic receptors on projecting neurons of the corpus striatum. (Felder, Bymaster et al. 2000) The M4 subtype, together with the M2 receptors, account for the muscarinic anti-nociceptive effects both in spinal and supraspinal sites. (Duttaroy, Gomeza et al. 2002) They are located prejunctionally on cholinergic nerve terminals of the urinary bladder, along with the M2, and inhibit the release of transmitters. (Chess-Williams 2002)

The M5 subtype is abundant in several structures of the brain, including the ventral tegmental area, which provides major dopaminergic innervation to some limbic areas, and the compact part of substantia nigra, where it is the only muscarinic subtype present

in this structure providing the main dopaminergic innervation to the striatum. (Vilaro, Palacios et al. 1990) It seems to facilitate striatal dopamine release along with other muscarinic subtypes, including the M4. (Eglen and Nahorski 2000) The M5 receptor has been shown also in various non-neuronal tissues, e.g. fibroblasts and keratinocytes, lymphocytes, endothelial cells and smooth muscle of the neurovasculature. (Buchli, Ndoye et al. 1999) It is also present in the salivary glands (Ryberg, Warfvinge et al. 2008) and in the eye on the iris sphincter (Gil, Krauss et al. 1997). The M5 receptor has so far been difficult to study because of the lack of selective M5 agonists; in particular it is very hard to distinguish between M3 and M5 mediated responses. Also no tissues in which the M5 receptor was the dominant muscarinic receptor subtype have been identified. (Ito, Oyunzul et al. 2009)

2.3 Salivary glands

Saliva plays an important role in many physiological functions, such as lubrication of the oral mucosa, aid for swallowing and speech, promotion of remineralization of the teeth, and protection against microbial infections due to the bactericidal effect of lysozyme. Although saliva plays a minor role in digestion, it helps prepare the food bolus for swallowing and is a solvent for the tasteants which delivers them to taste buds. Saliva is hypotonic compared to the plasma and it is also alkaline, which is important for the neutralization of any eventual acid reflux.

Saliva is produced by the three pairs of major salivary glands and numerous minor glands (e.g. labial, palatal and buccal), which are located throughout the upper aerodigestive tract, mainly in the oral cavity and the oropharynx. The major salivary glands are the parotid, submandibular and the sublingual glands.

2.3.1 Anatomy and histology of the salivary glands

The parotid glands are the largest salivary glands and they are located anteroinferior to the ear, overlying the mandibular ramus and masseter muscle. They extend medially between the mandibular ramus and the temporal bone to occupy the parapharyngeal space. The substance of the glands is divided into superficial and deep lobes by the

facial nerve. (Lalwani 2008) The drainage of the secretion of both glands is provided by a parotid duct, which empties into the vestibule.

The sublingual salivary glands are covered by the mucous membrane of the anterior floor of the oral cavity. A number of sublingual ducts open along both sides of the lingual frenulum. (Martini, McKinley et al. 2000)

The submandibular glands are located in the submandibular triangle together with lymph nodes and branches of the facial vein and facial artery. The hypoglossal, lingual and marginal mandibular nerves are all closely connected with the submandibular gland.

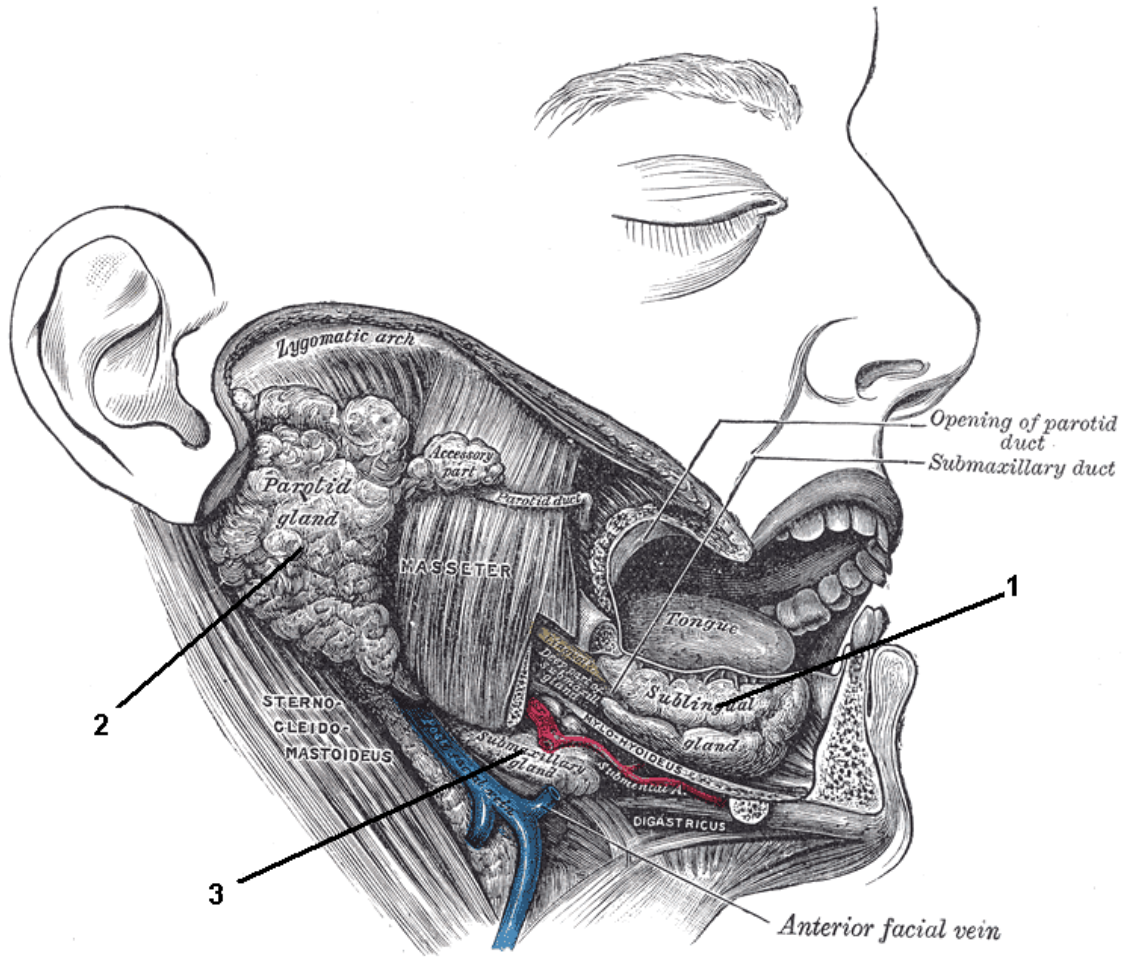

Fig. 2. Anatomy of the salivary glands

1 – Sublingual gland, 2 – Parotid gland, 3 – Submandibular gland

Adapted from: Gray's anatomy, http://www.bartleby.com/107/242.html#i1024

Histologically, the salivary glands are built up of lobules divided by septa of connective tissue. Each lobule is covered by a connective tissue capsule and consists of the acini, intercalated ducts, and small striated ducts. Larger striated ducts and excretory ducts are situated inside the connective tissue septa. The saliva produced by the secretory cells of the glands is carried through the tree-like network of these fine ducts which assemble into a central draining duct. This duct then penetrates the capsule and ends on the surface of the oral mucosa.

The acinus forms the distal part of the salivary unit. It consists of pyramidal secretory cells and myoepithelial cells intercalated between the basal sides of the secretory cells and the basement membrane. Acinar cells may be serous, mucinous, or seromucinous, which explains the different chemical compositions of the saliva of each gland. (Lalwani 2008) The parotid gland, for example, is dominated by serous secretory cells and produces thick secrete which contains a lot of enzymes, namely amylase, the first enzyme in the process of complex carbohydrates breakdown. The sublingual gland is dominated by mucous secretory cells and produces saliva rich in mucins. The submandibular gland contains both cell types and produces both type of saliva. The minor salivary glands mostly consist of seromucinous cells. The saliva in the mouth is a mixture of glandular secretions; about 70 % of the saliva originates in the submandibular glands, 25 % from the parotid gland and 5 % from the sublingual gland. All together, the salivary glands produce 1.0 – 1.5 litres of saliva a day, with the composition of 99.4 % water and various ions, metabolites, buffers and enzymes. (Martini, McKinley et al. 2000)

2.3.2 Physiology and regulation of secretion of the salivary glands

The primary isotonic fluid of the saliva is produced in the acinary cells, which are water-permeable, in contrast to the ductal cells. As the forming saliva travels through the ducts, most Na^+ and Cl^- are reabsorbed and a small amount of K^+ and HCO^{3-} are secreted. Since the duct epithelium has low hydraulic conductivity, the reabsorbed ions are not followed by water and this results in hypotonic secretion. The final composition of the serous secretion varies with the rate of the flow; the higher the rate, the closer the ionic composition is to that of primary secretion. Increases in the flow of saliva may be caused by the stimulation of receptors in the mouth, pharynx and oesophagus.

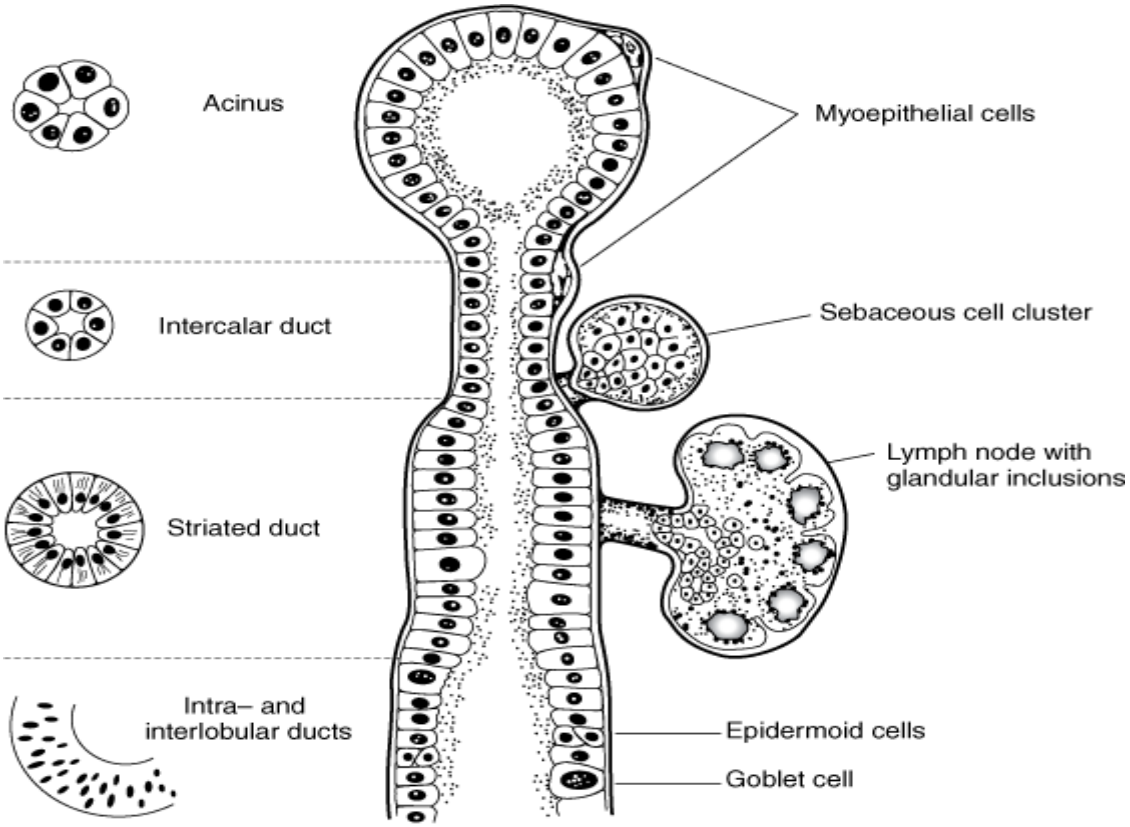

Fig. 3. The salivary gland unit

Adapted from: Lalwani, A. K. (2008). Current diagnosis & treatment in otolaryngology- Head & neck surgery. New York, McGraw-Hill Medical.

Salivation is a reflex action and is completely under neural control. Afferent sensory fibres conduct information to salivary centres in the pons and medulla of the brain and efferent autonomic fibres innervate the glands. The salivatory centres also proceed impulses from higher brain centers. The salivatory response is a good example of the parasympathetic and sympathetic systems not acting as mere antagonists. Instead, here they have a similar, but not identical effect. Parasympathetic stimulation, being the most important, causes a prolonged abundant secretion relatively poor in protein and vasodilatation, which is due to the release of VIP, a co-transmitter in numerous parasympathetic postganglionic nerves. Nitric oxide also contributes significantly to vasodilatation. An indirect effect of the M1 receptor on arterial smooth muscle

relaxation was proposed via influencing the synthesis of NO. (Ryberg, Selberg et al. 2008) Sympathetic stimulation produces a small quantity of thick mucous saliva, which is accompanied by vasoconstriction. (Petersen 2007) However, blood flow is not an initial limiting factor for secretion, because the interstitional fluid preserves the response for some time, until the intravascular oncotic pressure rises so much that the salivation lessens, unless the blood flow is increased. (Lung 1998; Thakor, Brown et al. 2003)

The acetylcholine-mediated increase in the salivary secretion was originally imputed just to the muscarinic M3 receptor, but later binding and molecular experiments indicated the expression and functional role of muscarinic M1 receptor, and sometimes also the M5 receptor. It has been shown that in order to reach maximal glandular response, both M1 and M3 muscarinic receptors need to be activated concordantly. The M1 receptor seems to be of particular significance at low intensity of stimulation. (Tobin, Ryberg et al. 2006) Thus, the M3 receptors predominantly mediate salivatory response and are included in the control of saliva volume and secretions of both high and low viscosity, whereas the M1 receptor is included in the control of high-viscosity lubrication. (Abrams, Andersson et al. 2006) Muscarinic M4 and M5 receptors also seem to contribute to the secretion, based on mice knockout experiments, where the buccal cavity of M3-knockout mice remained lubricated. (Takeuchi, Fulton et al. 2002; Bymaster, McKinzie et al. 2003)

On the molecular level, the M3 receptor mediates the salivatory signal of acetylcholine binding by activating a pathway that results in the production of 1,4,5-IP_3, which binds to and opens the 1,4,5-IP_3 receptor on the endoplasmatic reticulum, leading to the release of Ca^{2+}. This stimulates the Ca^{2+}-induced release of Ca^{2+} via the 1,4,5-IP_3 receptor and the ryanodine receptor (activated by cyclic ADP ribose). Increased intracellular Ca^{2+} activates the Cl^- channel embedded in the apical membrane, and the basolateral K^+ channel. Efflux of Cl^- to the lumen od the acinar cell draws Na^+ across the cell in order to maintain electrochemical neutrality, resulting in the osmotic gradient- generated fluid secretion. (Tobin, Giglio et al. 2009)

Prejunctional muscarinic receptors may alter parasympathetic signal transmission. Muscarinic M1 receptors usually facilitate the release of transmitter when the nerve activity is intense and short. (Tobin 1998) However, M2 and perhaps M4 receptors inhibit acetylcholine and peptide-mediated transmissions at low frequencies, but only

after some delay. (Tobin, Ryberg et al. 2006) Moreover, it was observed that a phenomenon of prominent enhancement of vasodilatation and secretion when compared with continuous stimulation occurs when the parasympathetic innervation is stimulated in burst pattern at high frequencies. This has been ascribed to the neuropeptides, which are preferetially released at high frequencies (Andersson, Bloom et al. 1982), and to the finding that short-lasting stimulation activates prejunctional facilitator and not inhibitory mechanisms. (Tobin 1998)

As for the tissue localization of the muscarinic receptor subtypes, according to the findings of Ryberg based on immunohistochemical staining on rat and ovine salivary glands, immunoreactivity for all of the muscarinic receptors was detected in the acini, except for the rat sublingual gland. A weaker occasional immunoreactivity for different muscarinic receptor subtypes was present in the ducts of different glands as well. M3 receptors were expressed in the intraglandular vessels of rat submandibular and parotid glands. The M4 receptors were found on nerve fibres at the outer layer of the lobuli and the submandibular vessels also expressed M1 and M2 receptors, in contrast to the parotid gland. Occasionally, M5 receptors were apparent in the arteries and veins as well. (Ryberg, Warfvinge et al. 2008)

2.4 Lacrimal gland

2.4.1 Function of the tears

Tears form a complex thin film of fluids that continuously covers the exposed surfaces of the eye, the cornea and the conjunctiva. Tears have various functions which are crucial for preserving the health of the eye. They provide optimal pH, nutritients, proteins, electrolytes, lipids and mucin for the nourishment and enhancement of transparency of the avascular cornea. They protect the cornea and the conjunctiva from harmful environmental stresses, such as cold, bright light, decreased humidity, physical injury, mechanical stimulation, chemicals and bacterial, viral, or parasitic infections. The latter is achieved by proteins, namely lysozyme and lactoferin. The tight neural regulation allows the tear secretion to respond immediately to these changing conditions. Although there are multiple pathways to stimulate lacrimal gland secretion, defects can appear and decrease the amount or change the composition of the tears, thus

resulting in dry eye syndromes, which can seriously deteriorate the quality of life and even threaten normal vision. (Hodges and Dartt 2003)

2.4.2 Anatomy and histology of the lacrimal gland

The main lacrimal gland is almond-shaped and is located within a depression in the frontal bone, at the roof of the orbit, superior and lateral to the eyeball. Anteriorly, it is divided into an upper (orbital) and lower (palpebral) lobe which is connected to the lateral superior conjunctival fornix by approximately 12 secretory ducts opening to the conjunctival sac (this applies to the human lacrimal gland, but the rat lacrimal gland is exorbital and secretes into a single long excretory duct). Structurally, it is similar to other exocrine ducts, as it consists of numerous small lobules made up of many fine branched tubules. These tubules consist of the actual secretory cells, the acinar cells, representing 80% of the mass of the gland. The tubules converge, forming interlobular ducts and then empty to the larger secretory ducts leading to the ocular surface. The secretory product is modified on its way through the ducts by the ductal cells.

The acinar cells are highly polarized due to the tight junctions surrounding the cells at the lumen and thus dividing the plasma membrane into apical (towards lumen) and basolateral (towards blood). This organization allows the secretion to flow in only one direction. The basolateral part contains receptors for the neurotransmitters and neuropeptides released from the proximate nerve endings, and transport proteins and ionic channels for the electrolyte- and water secretion. The cuboidal cells lining the ducts are also polarized and contain luminal tight junctions. Their main function is to secrete water and electrolytes, but they also secrete a limited amount of proteins. (Hodges and Dartt 2003)

The acinar and ductal cells are surrounded by the contractile myoepithelial cells, which are stellate with numerous thin radial processes ended by freely terminated tapered endings. (Nagato, Yoshida et al. 1980) The functional role of these cells is probably to support the secretory processes by contraction of their multiple processes on the basal side. They may also have structural role supporting the gland's shape. The lacrimal gland also contains a variety of components of the immune system, such as lymphocytes, plasma cells, mast cells and macrophages.

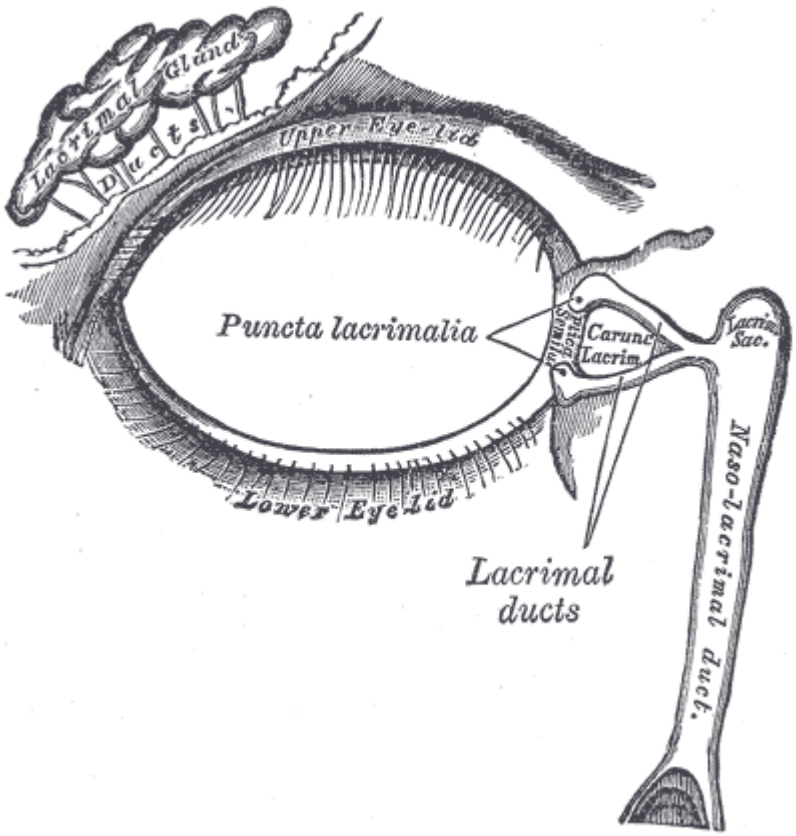

Fig. 4. The lacrimal apparatus. Gray's Anatomy of the Human body

Adapted from: http://www.bartleby.com/107/illus896.html

The tear film consists of three layers, each of them are produced by different structures. The inner mucous layer is produced by the goblet cells of the conjunctiva and stratified squamous cells of the cornea and conjunctiva. This helps to prevent bacterial binding and also has a smoothing effect. The middle aqeous layer is secreted principally by the main lacrimal gland, and the much smaller accessory lacrimal glands of Krause and Wolfring situated in the stroma of the orbital conjunctiva. The outer lipid layer is a product of the Meibomian glands, which can be found at the rim of upper and lower eyelids. The main function of this layer is to prevent evaporation of the tears. The rate of tear production is about 1 ml/day.

Protein secretion of the lacrimal gland can be divided into regulated and constitutive. Regulated secretory proteins are stored in secretory granules, which fuse with the apical plasma membrane immediately after stimulation by classic neurotrasmitters, bioactive peptides or peptide hormones. Granules containing constitutive secretory proteins are

not stored, but fuse with the plasma membrane without need for stimulation. Steroid hormones control the rate of their synthesis. (Hodges and Dartt 2003)

2.4.3 Innervation of the lacrimal gland

Lacrimal gland secretion is under hormonal and neural control. The innervation is provided by the lacrimal nerve (sensory- afferent), the facial nerve (parasympathetic-efferent) and the sympathetic nerve fibres, which arise from the superior cervical ganglion. The lacrimal nerve carries sensory fibers from the lacrimal gland, and it is one of the three branches of the opthalmic division of the trigeminal nerve. (Tasman 2007) The parasympathetic fibres are the most densely distributed and contain acetylcholine and VIP. They surround the basolateral membranes of most of the acini. (Dartt, Baker et al. 1984) The sympathetic nerves which release norepinephrine as a mediator, end at some acini and blood vessels. They are present to a much less extent than parasympathetic fibres, though. Sensory nerves are much less dense than the autonomic nerves and contain calcitonine gene-related peptide (CGRP) and substance P. (Nikkinen, Lehtosalo et al. 1984)

2.4.4 Receptors in the lacrimal gland

Secretion from the lacrimal gland is mainly stimulated by acetylcholine, acting on muscarinic receptors; VIP, acting on VIP1 and VIP2 receptors, and norepinephrine, stimulating α1 and β1 receptors. Muscarinic receptors are present on the basolateral membranes of acinar cells and the plasma membranes of myoepithelial cells, as it was shown by immunofluorescent labeling on the collagenase-digested acini preparations. (Lemullois, Rossignol et al. 1996) Hodges claims only the M3 receptor to be present in the lacrimal gland. (Hodges and Dartt 2003) However, the papers on which this statement is based are not completely convincing. Hootman conducted experiments on the avian salt glands (Hootman and Ernst 1981), and Mauduit characterized the muscarinic receptors only by pharmacological tools, using 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP), which antagonizes the M1/M3/M5 receptors and therefore couldn't definitely distinguish between M3 and M5 receptors. In Northern blotting, no

probes for M4 or M5 receptors were used. Also no further molecular methods were carried out. (Mauduit, Jammes et al. 1993). This means that further investigation of the muscarinic network in the lacrimal glands should be carried out.

However, in contrary to other subtypes, the presence and the functional role of the M3 receptor is well established. The receptor interacts with a $G_{q/11}$ protein upon activation, which leads to production of 1,4,5-IP_3 and DAG, as described before. Binding of 1,4,5-IP_3 to its receptors on structures such as the endoplasmatic reticulum leads to the release of Ca^{2+} into the cytosol. This process seems to be biphasic and the immediate release of Ca^{2+} from intracellular stores is followed by a slower sustained phase caused by Ca^{2+} entry across the plasma membrane. (Bird, Rossier et al. 1991) The activation of Ca^{2+} entry into the cell caused by Ca^{2+} release from the intracellular stores is called „the capacitative calcium entry“. (Berridge 1995) There are several theories to explain the mechanism. The first put up that the Ca^{2+} decrease in intracellular stores triggered the release of a substance that activated the capacitative calcium channels of the membrane. (Randriamampita and Tsien 1993) However, this substance hasn’t been identified yet. The second proposed a model of intracellular calcium stores-depletion regulated insertion of voltage-independent channels into the plasma membranes. (Fasolato, Hoth et al. 1993) This is referred to as the exocytosis model. The third theory involves conformational coupling between proteins on the intracellular stores and the capacitative Ca^{2+} entry channels. (Berridge 1995) The results of Sundermeier and Satoh obtained on single acinar cells indicate that the released Ca^{2+} from intracellular stores evoked fusion of secretory vesicles with the plasma membrane and leaded to exocytotis of secretory proteins. (Satoh, Sano et al. 1997; Sundermeier, Matthews et al. 2002) Ca^{2+} entering through the capacitative channels is probably responsible for long-term protein secretion; this is supported by the results of chelation of the intracellular Ca^{2+}, which led to inhibion of the initial rapid secretory phase, but was much less effective on prolonged secretion. (Hodges, Dicker et al. 1992)

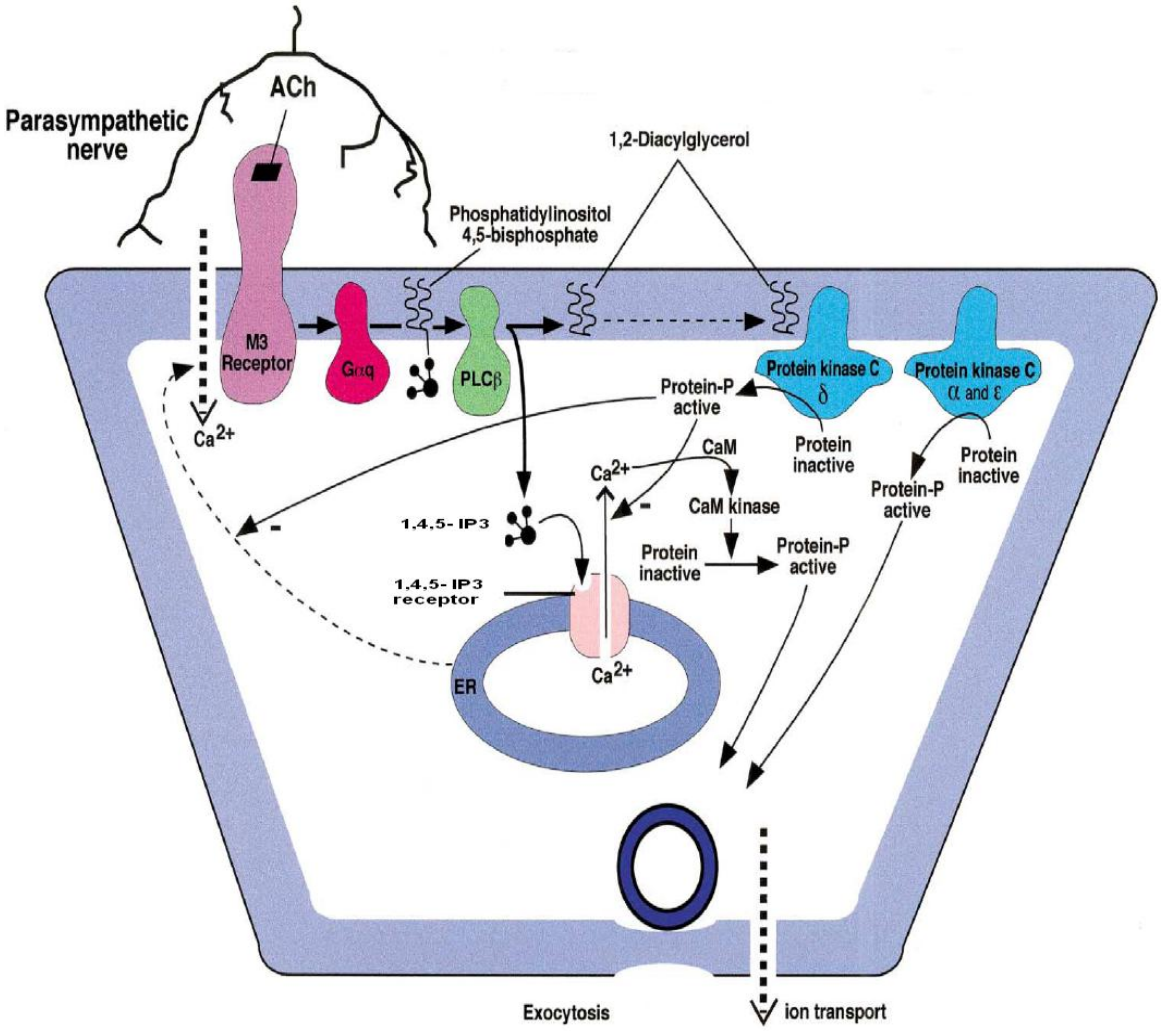

Fig. 5. Schematic drawing of pathways activated by cholinergic agonists in the lacrimal gland to stimulate protein secretion.

*Adapted from Dartt, D. A. (2001). "Regulation of lacrimal gland secretion by neurotransmitters and the EGF family of growth factors." Exp Eye Res **73**(6): 741-52.*

DAG leads to activation of the enzyme family called protein kinase C, which includes 11 different isoforms, each of these having cell-and tissue-specific localizations and functions. These enzymes phosphorylate (and in this way activate) protein substrates on serine and threonine residues. They may play a role in cholinergic activation of lacrimal gland secretion. PKC –α seems to induce protein secretion, while PKC -δ and –ε probably inhibit Ca^{2+} influx through the capacitative channels and thereby terminate secretion. (Hodges and Dartt 2003)

Cholinergic agonists also lead to activation of phospoholipase D besides PLC. PLD hydrolyzes phosphatidylcholine and produces a free polar head group and phosphatidic acid, which may be degraded to generate DAG or may serve as a signalling molecule itself. However, the role of PLD in lacrimal gland secretion has not been determined yet. (Hodges and Dartt 2003)

However, it has been shown that acetylocholine doesn't exclusively stimulate secretion. Cholinergic agonists may also activate a pathway which actually supresses the secretory response. This is achieved through activation of mitogen-activated protein kinase (MAPK) pathway, by increased levels of intracellular Ca^{2+}, activaton of PKC and subsequent activation of non-receptor tyrosine kinases. (Hodges, Rios et al. 2006) This finding was surprising, as activation of MAPK pathway is usually stimulatory. The purpose of this inhibitory pathway may be to regulate the amplitude of the secretory response. (Dartt 2009)

2.4.5 Dry eye syndrome (keratoconjunctivitis sicca)

Generally speaking, dry eye syndrome is a tear film abnormality caused either by unsufficient tear production or altered composition of the tear fluid, leading to excessive tear evaporation. Thus, the syndrome can be divided into two major types: aqeous-defficient dry eye caused by lacrimal gland diseases and evaporative dry eye caused mainly by diseases of the Meibomian glands. (Zoukhri 2006) Types of tear-deficient dry eye include Sjögren's syndrome, lacrimal disease, lacrimal obstructive disease, and reflex hyposecretion. Evaporative dry eye occurs even when the lacrimal function is normal and tear volume is sufficient. It can be caused by periocular disorders that interfere with tear film integrity, such as blepharitis, meibomian gland disease, and abnormalities with blinking. (McCabe and Narayanan 2009)

Under several pathological circumstances, the lacrimal gland can become a target of the immune system and get inflammed as a result of autoimmune diseases (such as Sjögren's syndrome), graft versus host disease after bone marrow transplantation, or as a result of aging. This leads to insufficient tear secretion and subsequently dry eye syndrome.

Millions of people all around the world suffer from the dry eye syndrome and mostly women and elderly people are affected. The patients mostly complain of scratchy or

sandy foreign body feeling, itching, excessive mucus secretion, inability to produce tears, a burning sensation, photosensitivity, redness, pain, and difficulty in moving the lids. Advanced forms of the syndrome may even lead to corneal ulceration, corneal thinning and perforation. Secondary bacterial infection may also occur, and corneal scarring and vascularization may result in significant impairment of vision. The treatment is usually restricted to alleviation of symptoms using artificial tear eye drops to replace the missing fluid.

2.5 Inflammation

2.5.1 Mechanisms of accute and chronic inflammatory response

Inflammation is a non-specific primary reaction of living tissue to injury, which can be due to many factors, such as mechanical, physical and biological. Symptoms and mechanism of inflammation should be considered after division into acute and chronic.

The acute inflammatory response lasts from minutes to few days and is characterized by the classic symptoms of rubor, calor, tumor and dolor, which have been recognized for thousands of years. Rubor and calor, or redness and heat, are caused by the increased blood flow and vascular permeability, leading to accumulation of protein-rich exudate and swelling (tumor). Dolor, pain is caused by stimulation of the nerve endings in the affected area by activated plasma kinins and histamine, which is being released from mast cells and basophils during the process. In response to injury, chemotaxins are being released from the damaged tissue, the microorganisms or as a product of complement-activation. This causes the leukocytes to migrate from the circulation into the affected area. The cells involved in the process of acute inflammation are neutrophils and macrophages, which are being transformed from monocytes when entering the tissue. The primary function of the macrophages is to phagocyte microorganisms and to remove remains of the damaged tissue. (Petersen 2007) Macrophages in addition produce TNF (tumour necrosis factor), which accounts for the main clinical symptoms of inflammation, and intensifies and prolongs the inflammatory response by activating other cells to release pro-inflammatory cytokins and mediators. (Tracey 2002) The immune system also produces antiviral interferons, derivatives of the

complement cascade and antibacterial and antitoxic antibodies, which may be found in the exsudate, thus contributing to inflammation. (Petersen 2007)

A widely used clinical marker for acute inflammation, C-reactive protein (CRP), plays several roles in the inflammatory process, including initiation of opsonization and phagocytosis and activation of complement, neutrophils, and monocytes. Its levels may increase significantly in 24 – 48 hours as high as 1000-folds. (McClatchey 2002)

The chronic inflammatory response is a highly regulated process that can last for weeks or months, sometimes even years. Mononuclear cells, such as lymphocytes, monocytes and plasma cells play the most important role. In contrast to acute inflammation, the chronic inflammatory response is characterized by proliferiation of capillaries and increased deposition of extracellular matrix components, which is essential for wound healing and repair. It can be triggered by persistent infection with a variety of microorganisms, long term exposure to insoluble exogenous particles, or by autoimmune diseases. The characteristics of the chronic inflammatory responses depend on the location and cause of the injury. The key cell type in the process are the macrophages that can be activated by γ-interferon secreted by antigen-activated T lymphocytes and microbial endotoxins, extracellular matrix proteins, or foreign particles. When activated, they enhance their phagocytal activity and secrete a wide variety of mediators, such as proteases, reactive oxygen and nitrogen intermediates, coagulation factors, arachidonic acid-derived lipids, cytokines, angiogenic factors (e.g. fibroblast growth factor), and profibrogenic growth factors (fibroblast growth factor, transforming growth factor-β, platelet-derived growth factor). The lymphocytes, both T and B cells are also important. They are recruited by the same chemotactic mechanisms as neutrophils in acute inflammation, and also by leukocyte-endothelial adhesive interactions. Activated lymphocytes produce a wide range of proinflammatory mediators that are involved in immune regulation (e.g., IL-5 in IgE) and in lymphocyte proliferation (e.g. IL-2). (Williams and Lichtman 2006)

2.5.2 Regulation of the inflammatory process

The mechanisms that regulate the process of inflammation include mediators of both humoral and neural origin and their aim is to maintain the inflammatory response within physiological range. (Rosas-Ballina and Tracey 2009) Glucocorticoids, IL-10 and other

humoral anti-inflammatory mediators hold back the release or the effect of proinflammatory cytokines, while resolvins and lipoxins assist tissue healing. (Serhan and Savill 2005) Humoral mediators affect not only target cells but also other organs, in contrast to nerve-released mediators, such as norepinephrine and acetylcholine, which are targeted and act almost immediately. It was found out that the afferent portion of the vagus nerve, the major parasympathetic nerve, also transfers information about inflammatory processes in the periphery to the brain, while the efferent portion of the vagus nerve affects the inflammatory response. This mechanism is known as the inflammatory reflex. The efferent part of the inflammatory reflex, the cholinergic anti-inflammatory pathway is composed of the efferent vagus nerve, acetylcholine and the α7 subunit of the nicotinic acetylcholine receptor. (Rosas-Ballina and Tracey 2009)

Borovikova has shown that acetylcholine significantly reduced the release of cytokines (TNF, IL-1β, IL-6 and IL-18), but not the anti-inflammatory cytokine IL-10, in lipopolysaccharide-treated human macrophage cultures. (Borovikova, Ivanova et al. 2000) This indicates that acetylcholine only inhibits the production of pro-inflammatory cytokines. The α7 subunit of the nicotinic acetylcholine receptor plays a key role in the pathway, as it links the nervous and the immune systems. (Rosas-Ballina and Tracey 2009)

However, a role for lymphatic muscarinic receptors has been proposed in the immune response as well. Muscarinic receptors have already been reported to mediate autocrine, non-neuronal functions of acetylcholine in various tissues. These functions are generally different from the roles of acetylcholine in neurotransmission and they include regulation of basic cell functions, such as differentiation, cell growth and apoptosis. (Eglen 2006) Lymphocytes have been shown to express various cholinergic components, including muscarinic receptors, where acetylcholine acts in autocrine manner. Lymphocytes have been shown to express all five subtypes of muscarinic receptors, both on mRNA and protein level. Activation of M1, M3 or M5 receptors is probably involved in modulating immune responsiveness, because increases in intracellular Ca^{2+} were detected after agonist stimulation of T and B lymphocytes. (Kawashima and Fujii 2003). Later on, it was shown that the secretion of IL-6 was markedly reduced in immunized M1/M5 knockout mice, suggesting that these receptors modulate antibody class switching from IgM to IgG_1 via cytokine production. However, there was no change was detected in the serum IgM level, so they probably don't take

part in the initial generation of the antibody response. (Fujii, Tashiro et al. 2007) Nomura suggested a connection between the activation of M1 receptors and IL-2 production and thus positive modulation of cell growth in human T lymphocytes (Nomura, Hosoi et al. 2003) and the M1 receptor has also been shown to play a role in the in the differentiation of $CD8^+$ into cytolitic T lymphocytes in in vitro experiments on mice (Zimring, Kapp et al. 2005). Fujii reported also that stimulation of either T- or B-lymphocytes with their correspondent mitogen leads to up-regulation of M5 muscarinic receptor mRNA expression, suggesting that direct interaction between T-lymphocytes and B-lymphocytes or antigen presenting cells leads to up-regulation of cholinergic transmission in lymphocytes via M5 receptors. (Fujii, Watanabe et al. 2003) The exact physiological function of this receptor is not exactly known, but it has been shown to be coupled to nitric oxide synthase (Wang, Zhu et al. 1994) so it may be involved in the regulation of production of NO in lymphocytes. (Costa, Auger et al. 1995)

Muscarinic receptor subtypes were also reported to intermediate the inflammation-influencing effect of acetylcholine within different organs. (Pavlov and Tracey 2006) The M3 subtype was characterized in the rat tracheal epithelium to mediate the release of prostaglandin E_2 and activation of phospholipase A2. (Brunn, Wessler et al. 1995) Acetylcholine has also been reported to release lipoxygenase-derived neutrophil and monocyte chemotactic activity via the M1 receptor in the bronchial epithelium during airway inflammation. (Koyama, Rennard et al. 1992) M3 and M5 receptors were shown to stimulate differentiation of cultured inflammatory cells into monocytic/macrophagic cells. (Mita, Dobashi et al. 1996) This manifests the somewhat contradictory effects of muscarinic receptors on inflammation, as the central muscarinic receptors have an activating role in the regulation of the cholinergic anti-inflammatory pathway. (Pavlov, Ochani et al. 2006) Muscarinic receptors seem to take part in the remodelling processes occuring during chronic inflammatory diseases, asthma being an example. (Gosens, Bos et al. 2005) According to Budd, the M1, M3 and M5 muscarinic receptors may mediate protection against apoptotic cell death. (Budd, McDonald et al. 2003) The M2 receptor has been shown to trigger the production of the bioactive sphingolipid sphingosine-1-phosphate (S1P). (Pfaff, Powaga et al. 2005) S1P stimulates proliferation of airway smooth muscle and release of cytokines, thus posesses inflammatory and proliferatory features. (Ammit, Hastie et al. 2001) However, the activation of muscarinic receptors on its own doesn't induce proliferation and cell growth; other co-acting pro-proliferatory

stimuli, such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) are requiered to achieve this. (Krymskaya, Orsini et al. 2000; Gosens, Nelemans et al. 2003)

2.6 Sjögren's syndrome

Sjögren's syndrome is named after the Swedish ophtalmologist Henrik Sjögren and it is a chronic inflammatory autoimmune and rheumatic disorder. It's primary form is characterized by lymphocytic infiltration of salivary and lacrimal glands, which leads to progressive xerostomia and xerophtalmia (so-called sicca complex) because of the lack of proper exocrine secretion of mucosal membranes. These invader cells are mostly CD^{4+} T-cells, which can produce IL-2, TNF α and other cytokines. (Fox and Kang 1992) One third of the patients develops the secondary syndrome with systemic manifestations such as arthritis, fever and fatigue and in this state the disease involves other organs, such as kidneys, lungs, liver, blood vessels and lymph nodes. This results in extraglandular pronouncements, including small-vessel vasculitis, glomerulonephritis and peripheral neuropathy. (Tucci, Quatraro et al. 2005) The secondary syndrome can be associated with almost any of the autoimmune rheumatic diseases, mostly rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis. (Jonsson, Moen et al. 2002) Patients with major salivary involvement are at an increased risk of developing non-Hodgin lymphomas of the parotid and submandibular gland. A minority of patients also suffers from high-frequency hearing loss of cochlear origin. (Tucci, Quatraro et al. 2005)

2.6.1 Prevalence and ethiology

Sjögren's syndrome occurs all around the world in all age categories and it's diagnosis must be confirmed by several blood, ophtalmologic and oral tests. Still, sometimes it takes years after the symptomes are onset to settle the final diagnose. Most of the patients are females between 40-50 years of age, with the female/male ratio 9:1, pointing to the role of sex steroid hormones. (Sullivan, Wickham et al. 1999)

The etiology is multifactoral. Genetic predisposition may prominently contribute to the development of the disease and also family history of other autoimmune diseases is common for the patients. Different viruses have been presumed to play a role in the ethiology of the syndrome as well, because the salivary glands may be a site of latent infections. (Jonsson, Moen et al. 2002) These include the Eppstein-Barr virus (EBV), hepatitis C virus (HCV), human T-cell leukemia virus (HTLV)-1 and human immunodefficiency (HIV)-1. (James, Harley et al. 2001) In addition, a relationship between Sjögren's syndrome and *Helicobacter pylori* has been proposed, as both these conditions are under increased risk of mucosa-associated lymphoid tissue lymphoma development. (Isaacson and Spencer 1987)

A large number of various autoantibodies have been found in both primary and secondary Sjögren's syndrome, and their presence can in some cases be in relation to the extent and severity of the disease. (Jonsson, Moen et al. 2002) These antibodies are aimed against organ- and non-organ- specific antigens. (Zoukhri 2006)

The autonomic nervous system may play an important etiologic role in the pathogenesis of Sjögren's syndrome, as it mimics several symptoms of ANS failure. The major symptoms of the syndrome, xerophtalmia and xerostomia suggest cholinergic parasympathetic dysfunction, whereas frequently reported xeroses and decreased sweating is a result of sympathetic cholinergic failure. (Nikolov and Illei 2009) Another important symptome, fatigue, can also be contributed to ANS dysfunction. (Barendregt, Visser et al. 1998) It's known that the degree of innervation is not changed (Pedersen, Dissing et al. 2000), so the ANS abnormalities may be possibly mediated through interference with signalling via muscarinic receptors. (Nikolov and Illei 2009)

2.6.2 Muscarinic receptors and Sjögren's syndrome

The discovery of serum M3 receptor autoantibodies was a significant step towards understanding the pathogenesis of glandular function deterioration. These antibodies simulate the effect of muscarinic receptor agonists and eventually they lead to the loss of the secretory funtion as they prevent proper parasympathetic neurotransmission. (Bacman, Sterin-Borda et al. 1996) According to the findings of Beroukas, the M3 muscarinic receptor expression is up-regulated in glandular acini in human labial glands. (Beroukas, Goodfellow et al. 2002) This was also confirmed by the results of

Ryberg, who reported up-regulation of M3, M4 and M5 receptors. (Ryberg, Warfvinge et al. 2008) The increased expression of M3 receptors is probably a result of long-term receptor blockade. (Tobin, Giglio et al. 2009) As for the M5 receptor, the up-regulation seems to be a common feature in inflammation. (Giglio, Ryberg et al. 2005)

2.6.3 Salivary glands and Sjögren's syndrome

The typical otolaryngological manifestations are xerostomia, which progressively makes swallowing and fluent speech difficult, burning oral sensation, frequent dental caries and chronic periodontitis. The mucosa of the oral cavity is dry, erythematosus and sticky. The lack of saliva can lead to atrophy of the tongue mucosa and mycotic infections. In addition, the progressive lymphatic infiltration of parotid glands is manifested by billateral swelling in half of the patients, and leads to ductal inflammation and acinar destruction. (Tucci, Quatraro et al. 2005) Another characteristic sign of Sjögren's syndrome in salivary glands is focal adenitis, which is found only in some of the labial salivary glands, and even there not in all the lobules. This is in contrast with the functional defect of the salivary glands. (Tornwall, Konttinen et al. 1997)

Loss of function of salivary glands is evaluated by measuring salivary flow rate (sialometry) and by chemical analysis of saliva (sialochemistry). According to Kalk, the saliva of the patients positive for Sjögren's syndrome had significantly changed composition in concentration of sodium, chloride, and phosphate and the flow rate of the submandibular and sublingual glands is fairly reduced, which may suggest their early involvement in the disease progression. (Kalk, Vissink et al. 2001)

For a long time it has been difficult to investigate the syndrome, due to the different criteria for the classification of the disease, and also partly because of the traditional approach to Sjögren's syndrome that the symptoms are a result of glandular tissue destruction. (Dawson, Smith et al. 2000) This interpretation of salivary glands pathophysiology was changed mainly by the findings that the glands of many patients comprise large amount of acinar tissue that is unfuctional in vivo (Humphreys-Beher, Brayer et al. 1999), but in vitro studies of isolated acinar cells from patients showed them to be functional, although with lower sensitivity to acetylcholine stimulation. This indicates that the salivary secretion impairment must not be necessarily caused by

immune-mediated acinar cell destruction. (Dawson, Field et al. 2001) It has also been shown that the glandular responsiveness is not significantly changed with age, even if larger amount of acinar tissue is lost. (Heft and Baum 1984) In conclusion, glandular function may be due to abberant acinar function, which may be followed by atrophy in the end. The discovery of antimuscarinic autoantibodies could therefore unite the pathologies of glandular hypofunction in both primary and secondary Sjögren's syndrome (Dawson, Tobin et al. 2005), as it was shown that there were no significant differences between the salivary flows in each clinical variant. (Dawson, Holt et al. 2001) The alternation of muscarinic receptor function by autoantibodies could also be responsible for extraglandular manifestations of Sjögren's syndrome, such as bladder irritability, among others. (Dawson, Tobin et al. 2005)

It was also proposed that the decreased secretory function may be caused by irresponsiveness of receptors, such as the α1-adrenergic, M1 and M3 muscarinic and NK-1 substance P receptors to activation. These are all coupled to protein-kinase C signalling pathway. The protein-kinase C family alters protein activity via serine/threonine phosphorylation. Their activation of PKC is essential for cell proliferation, differentiation, membrane transport, and gene expression. (Nishizuka 1992) Single cells usually co-express different isoforms of PKC. Long-term activation of the PKC system may result in proteolytic degradation and depletion, following the initial activation. It was shown that some isoforms are defficient in acinar epithelial cells, which may also lead to defect signal transduction. (Tornwall, Konttinen et al. 1997) These findings are in accordance with the presence of M3 autoantibodies, as they may lead to prolonged activation and depletion of some PKC isoforms.

2.6.4 Lacrimal gland and Sjögren's syndrome

The main ocular symptoms include dry eyes, characterized by burning, sandy and itchy sensations, redness of the conjunctiva and increased photosensitivity.

The lacrimal gland of patients with SS is vastly infiltrated by lymphocytes, but still they contain many healthy-looking acinar and ductal cells. However, the secretion of the tissue is significantly decreased, even if the remaining cell should produce enough tears to preserve the minimal tear layer, and this results in aqeous-defficient dry eye. This suggests that there may be an impairment in the neural innervation of the rest of

the healthy tissue. However, it was shown that there wasn't a noticeable change in the the density of parasympathetic innervation around the acini of Sjögren's syndrome model mice in the areas of normal acinar and ductal cells, demonstrating that the parasympathetic innervation is not changed with the progression of inflammation. This also applies for the sympathetic and sensory innervation. It was also found out that cellular response manifested by the increase in intracellular Ca^{2+} to exogenous stimuli is up-regulated, thus leading to denervation-like supersensitivity. (Zoukhri, Hodges et al. 1998) The altered stimulation of secretion may therefore be caused by the disabled release of transmitters from nerve endings, proinflammatory cytokines like IL-1 β being one of these inhibitory factors. (Zoukhri, Hodges et al. 2002) IL-1 β may induce nitric oxide synthase iNOS in lacrimal gland epithelial cells and therefore increase NO production, which may play a role in lacrimal cell death. (Beauregard, Brandt et al. 2003)

2.6.5 Treatment of sicca complex in Sjögren's syndrome patients

Currently, the treatment of most patients is restricted to alleviation of symptomes, including eye lubricants, saliva substitutes and pharmacological local and systemic stimulators of secretory functions. The main secretagogues are pilocarpine and cevimeline. Pilocarpine is a natural alcaloid, acts as a non-selective muscarinic agonist and was the first agent with demonstrable efficacy in this indication. (Mavragani and Moutsopoulos 2007) However, its usefulness is limited by its short action, necessitating frequent administration. Cevimeline is a quinuclidine derivative of acetylcholine that acts as a muscarinic agonist with higher affinity for the M3 receptor. Its half-life is longer than pilocarpine, so it can be administered three times daily and seems to improve the symptoms of dry mouth and the salivary flow rate significantly. (Weber and Keating 2008) Cevimeline has also been reported to be effective in improving dry eye symptomes in a clinical study, where it made a significant difference in subjective symptoms, tear dynamics and condition of the corneoconjunctival epithelium. (Ono, Takamura et al. 2004)

3 Aims

The aim of the present study is to investigate the presence of muscarinic receptor subtypes in the rat lacrimal and salivary glands, under normal and inflammatory circumstances. The questions concerned are:

1) Is the M3 subtype the only one present in the lacrimal gland, and if not, which other subtypes are present?
2) Are there any changes in expression of muscarinic receptor subtypes in healthy and inflammed lacrimal and salivary glands?
3) Is our model of inducing inflammation with LPS effective enough to induce changes in muscarinic receptor expression?
4) Are there any changes in expression of muscarinic receptor subtypes in healthy and focal adenitis human labial glands, which serve as a model for Sjögren's syndrome?

4 Materials and Methods

4.1 Immunohistochemistry

4.1.1 Introduction of the method

Immunohistochemistry (IHC) is the localization of antigens, e.g. proteins in tissue sections by the use of specific labeled antibodies through antigen-antibody interactions that are visualized by a marker such as enzyme or fluorescent dye. IHC is widely used in basic research to characterize the distribution and localization of biomarkers and differentially expressed proteins in distinct parts of a biological tissue.

Immunohistochemistry has apparent advantage over traditionally used enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures, as it involves specific antigen-antibody reaction. Therefore, IHC has become a crucial technique and is widely used in many medical research laboratories as well as clinical diagnostics.

There are numerous IHC methods that may be used to localize antigens. The selection of a suitable method should be based on parameters such as the type of investigated specimen and the degree of required sensitivity.

Tissue preparation is one of the most important steps in the immunohistochemistry procedure. The fixation has to be adequate and prompt to ensure the preservation of tissue architecture and cell morphology. However, there is no universal fixative that is ideal for the demonstration of all antigens. In general, many antigens can be successfully demonstrated in formalin-fixed, paraffin-embedded tissue sections. The discovery and development of antigen retrieval techniques further enhanced the use of formalin as routine fixative for immunohistochemistry. The most common fixatives used for IHC are 4% paraformaldehyde in 0.1M phosphate buffer, 2% paraformaldehyde with 0.2% picric acid in 0.1M phosphate buffer and 4% paraformaldehyde, 0.2% periodate and 1.2% lysine in 0.1M phosphate buffer (PLP fixative). However, certain cell antigens do not survive routine fixation and paraffin embedding, so the use of frozen sections still remains essential for the demonstration of numerous antigens.

The visualization of many antigens can be significantly improved by pretreatment with the antigen retrieval reagent that breaks the protein cross-links formed by formalin fixation and thereby uncovers hidden antigenic sites. The techniques involved the application of heat for varying lengths of time to formalin-fixed, paraffin-embedded tissue sections in an aqueous retrieval solution. So the retrieval of the epitopes may be induced by heat or alternatively by proteolytic enzyme digestion. Heat causes cross-linked protein epitopes to unfold and the buffer solution helps in maintaining the conformation of the unfolded protein. Microwave oven, pressure cooker and steamer are the most commonly used heating devices. Citrate buffer of pH6.0 is the most popularly used retrieval solution and is suitable for most of antibody applications. The TRIS-EDTA of pH9.0 and EDTA of pH8.0 are second most used retrieval solutions.

The next significant step of the IHC procedure is the blocking of non-specific background staining, as inadequate or delayed fixation or impure antigens used for immunization of the animal which produces the secondary antibody may give rise to false positive results. But usually the main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blocking of those sites with normal serum. Endogenous peroxidase activity is found in many tissues and can also be the cause of non-specific staining leading to false-positive results. The solution for eliminating endogenous peroxidase activity is pretreatment of the tissue section with hydrogen peroxide before the incubation with primary antibody.

As every valid scientific method, IHC also requires controls in order to test the protocol and the specificity of the antibody being used. Positive control is to test a protocol or procedure and make sure it works. Usually tissues of known staining results are used. If the positive control tissue showed negative staining, the protocol or procedure needs to be checked until a good positive staining is obtained. Negative control is to test for the specificity of an antibody involved. No staining must be shown when omitting primary antibody or replacing the specific primary antibody with normal serum.

Two methods, direct and indirect are commonly used in IHC. Direct method is one step staining method, and involves a labeled antibody reacting directly with the antigen in tissue sections. This procedure is short and quick, but it’s main disadvantage is insensitivity due to little signal amplification and therefore it is rarely used. The indirect

method involves an unlabeled primary antibody which reacts with tissue antigen, and a labeled secondary antibody reacting with primary antibody. It is very important that the secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised. This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. In the indirect immunofluorescent method, the secondary antibody is labeled by a fluorescent dye, while in the indirect immunoenzyme method, the secondary antibody is labeled by an enzyme, e.g. peroxidase. There are various versions of the indirect method, such as the PAP method (Peroxidase Anti-Peroxidase), LSAB method (Labeled StreptAvidin Biotin) or the ABC method (Avidin-Biotin Complex).

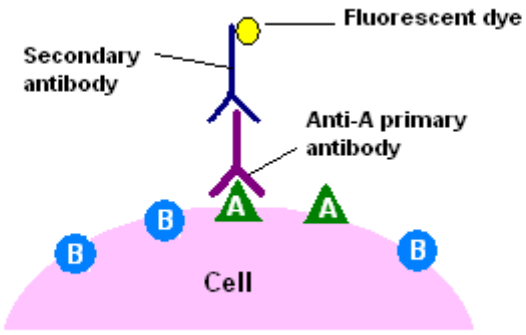

Fig. 6. Scheme of immunofluorescent labeling

The ABC method is a standard and widely used technique for immunohistochemical staining. Avidin, a large glycoprotein, can be labeled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies. The technique involves three layers. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. The peroxidase is then developed by the DAB (3,3'-diaminobenzidine) or other substrate to produce different colorimetric end products.

Adapted from: *http://www.ihcworld.com/introduction.htm*

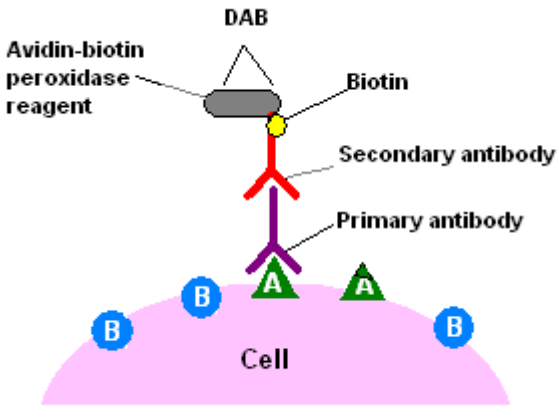

Fig. 7. Scheme of the ABC staining method

4.1.2 Immunohistochemistry procedure

The rat submandibular, parotid and lacrimal glands were used in this experiment. Male rats of the Sprague-Dawley strain with weight of 250-300 g were used in the current thesis. The study was approved by to local ethical committee of Göteborg University. The tissues were dissected out from the animal after the it had been anaesthetized by intraperitoneal injection of medetomidin (Dormitor, Orion Pharma, Finland) at a dose of 0.1 mg/kg and then was killed by carbon dioxide. The specimens were fixed in phosphate buffered 4 % paraformaldehyde (pH 7.0), and then embedded in paraffin. For the immunohistochemical investigation of muscarinic receptor expression, sections of different specimens were prepared in a cryostat at a thickness of 4 μm. These steps were done by HistoCenter, Västra Frölunda, Göteborg.

On every glass, there are two slices of the tissue, the left one on the plus side is used as the tested section, and the right one is used as negative control. This setup ensures that the controls and the tested specimens are processed under the same circumstances. The procedure itself takes two subsequent days. The first step is the de-paraffinization by heating the slides to 60 °C for 60 minutes and by immersing them into 100% xylene. Then they are re-hydrated by serial incubations in decreasing concentrations of ethanol, followed by running deionised water and tris-buffered saline (TBS) solution, which is used almost after every step to wash out the previously used solutions and to inactivate the ongoing reactions. Then the sections are immersed in 0.1 M citrate buffer (pH 6.0)

and microwaved to rupture the membranes and break protein cross-links. Endogenous peroxidase activity is blocked by 0.3 % aqueous solution of hydrogen-peroxide. Non-specific protein binding is then blocked with 5% goat serum in TBS (the goat serum was used instead of the usual bovine serum albumin, because we observed better staining results on the lacrimal gland, if the non-specific background is blocked by a serum of the animal in which the secondary antibody was produced). This is followed by the most important step of the day, the incubation with the primary antibody overnight at room temperature in a humidified chamber with polyclonal rabbit anti-mAChR subtype-specific antibodies raised in rabbit diluted in a ratio 1:100 in TBS containing 1% goat serum.

On the second day of the immunoenzyme ABC method, the specimens are incubated with biotinylated secondary antibody raised in goat of the Santa Cruz ABC Kit. It is very important to use the kit that is directed against the species of the primary antibody, which means against rabbit in this case. The following steps are the incubation with the AB enzyme reagent and subsequently with Peroxidase substrate reagent. Finally, the slices are counterstained with Mayer's haematoxyline, which selectively stains the cell nuclei with blue colour, after neutralizing the acid form in warm tap water to produce the insoluble blue complex of the reagent. Now that the staining is completed, the slices are gradually rehydrated in baths with increasing concentrations of ethanol and 100% xylene, then fixed with Pertex glue and covered by a coverslip.

The second day procedure of the immunofluorescenct method is different from the immunoenzyme method. The sections are incubated with fluorescently marked antibody. All the steps have to be carried out in darkness or at least shadow. Afterwards, the specimens are dehydrated in increasing concentrations of ethanol. Xylene was skipped in this method. After drying, ProLong medium is applied to the sections to preserve the signal for longer time. The sections are finally analysed by the use of fluorescence microscope.

4.1.3 Materials for the immunohistochemistry

Anti-muscarinic M1 and M5 receptor antibodies produced in rabbit (Research & Diagnostic Antibodies, USA); Anti-muscarinic M2 and M3 antibodies produced in rabbit (Sigma); Mayer's haematoxyline (HistoLab, Gothenburg, Sweden); Pertex (HistoLab, Gothenburg, Sweden); Goat serum (Sigma); AlexaFluor 488 goat anti-rabbit IgG (Invitrogen, USA); ProLong Gold antifade reagent (Invitrogen, USA); ABC staining system SC 2018 for use with rabbit primary antibodies containing blocking goat serum, biotinylated goat anti-rabbit IgG, avidin, biotinylated HRP, substrate buffer, peroxidase substrate and DAB chromogen (Santa Cruz Biotechnology, USA); Xylene (HistoLab, Gothenburg, Sweden); Ethanol 99.5%, 95%, 85%, 70% (Kemetyl, Sweden); Hydrogen peroxide (Sigma); Sodium citrate (Sigma), Citric acid (Sigma), Sodium chloride (Sigma), Trizma base (Sigma)

Microscope: Nikon Eclipse 90i

Camera: Nikon DS- Fi1

Magnification: Ocular: 10x Objective: 20x

Software: NIS-Elements D 3.10, Laboratory Imaging, Nikon

All pictures shown together and being compared in the Results part were taken under the same settings.

4.1.4 Inducement of inflammation in the salivary and lacrimal glands of rat by LPS

A male rat of the Sprague-Dawley strain with the weight of 260 g had been injected by a 10 µg/kg dose of buprenorfine (Temgesic, Schering-Plough) followed by an intraperitoenal injection of lipopolysaccharide at a dose of 100 µg/kg. LPS is a major component of the outer membrane of Gram-negative bacteria, acts as an endotoxin and elicits strong inflammatory responses in animals. The rat was kept in its cage with access to water and food ad libitum for 24 hours. After this the rat had been administered an intraperitoneal dose of medetomidin (Domitor, Orion Pharma, Finland) and was killed by carbon dioxide, the organs were dissected and kept in phosphate buffered 4 % paraformaldehyde. The organs showed macroscopical features of inflammation, such as swelling and redness. The tissue was sent for processing to the HistoCenter, Västra Frölunda, Göteborg.

5 Results

5.1 Immunohistochemistry of the lacrimal gland

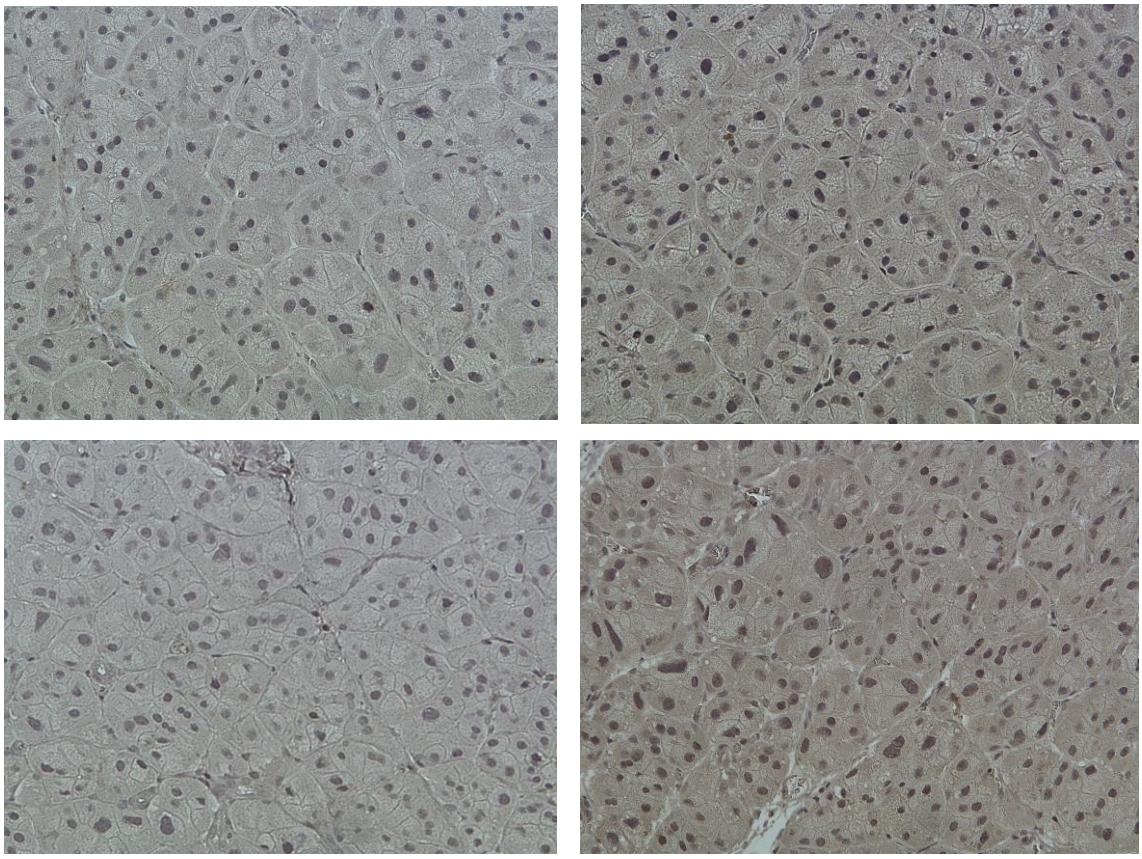

Fig. 8. Immunohistochemical labeling of the M1 muscarinic receptors in the rat lacrimal gland by immunoenzyme method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the staining for M1 receptors. All sections were counterstained with Mayer's haematoxyline.

Fig. 8. shows the results of immunohistochemical labeling of the M1 muscarinic receptors in the rat lacrimal gland. Positive results for the binding of the M1 antibodies on both pictures of the right column can be observed, manifested by brown staining. The pictures show the acini of the lacrimal gland, consisting of column-shaped serous cells organized in a circle. The acinar cells are polarized, which means that the nuclei and the receptors for the neurotransmitters are generally localised in the basolateral part, while the apical part is dominated by secretory granules. The acini are separated by thin layers of connective tissue. The myoepithelial cells cannot be distinguished in this view. The staining pattern shows specific brown staining on the basolateral sides of acinar the cells in both the healthy and LPS-treated tissue.

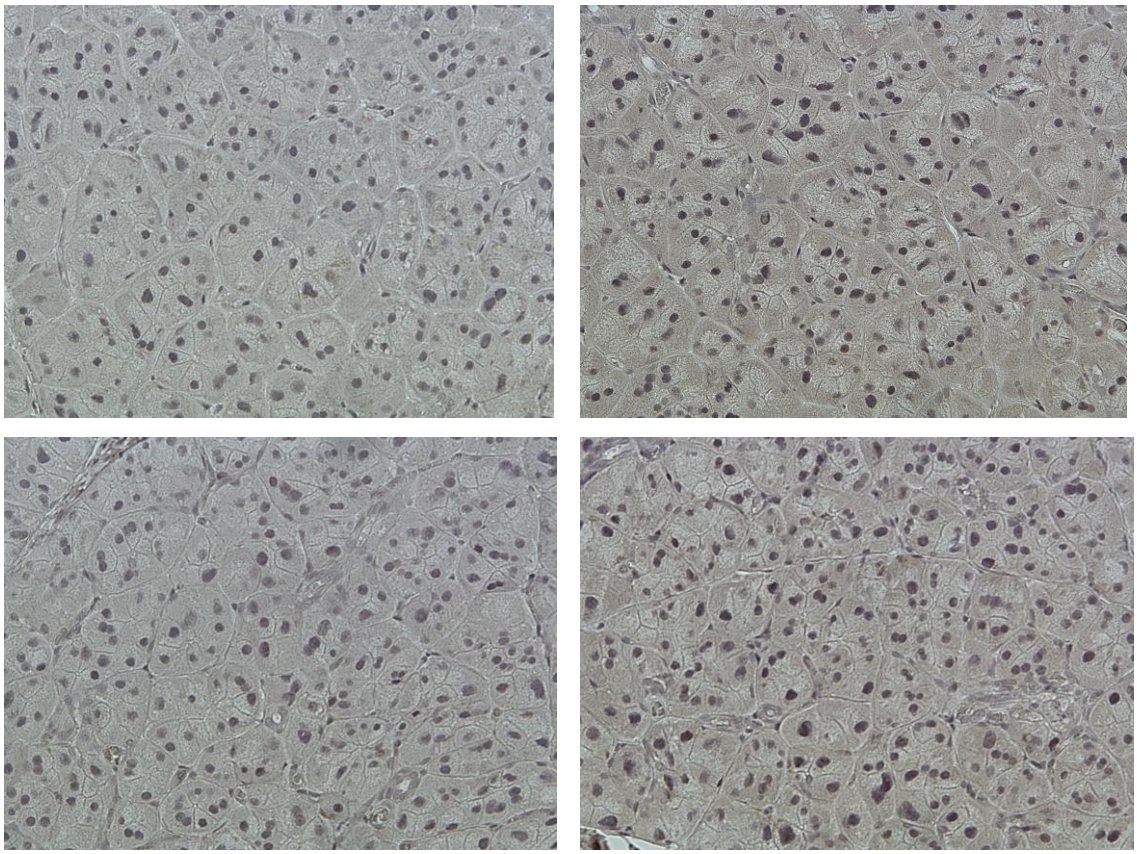

Fig. 9. Immunohistochemical labeling of the M3 muscarinic receptors in the rat lacrimal gland by immunoenzyme method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left side and the right side pictures show the staining for M3 receptors. All sections were counterstained with Mayer's haematoxyline.

Fig. 9. shows the results of immunohistochemical labeling of the M3 muscarinic receptors in the rat lacrimal gland. The same structures and staining pattern can be observed as in Fig.8., although the staining for the M3 receptor may seem a little bit weaker. The receptors are again localized in the right side pictures in the basolateral portions of the acinar cells. In these pictures any major differences between the normal and LPS-treated tissue cannot be observed.

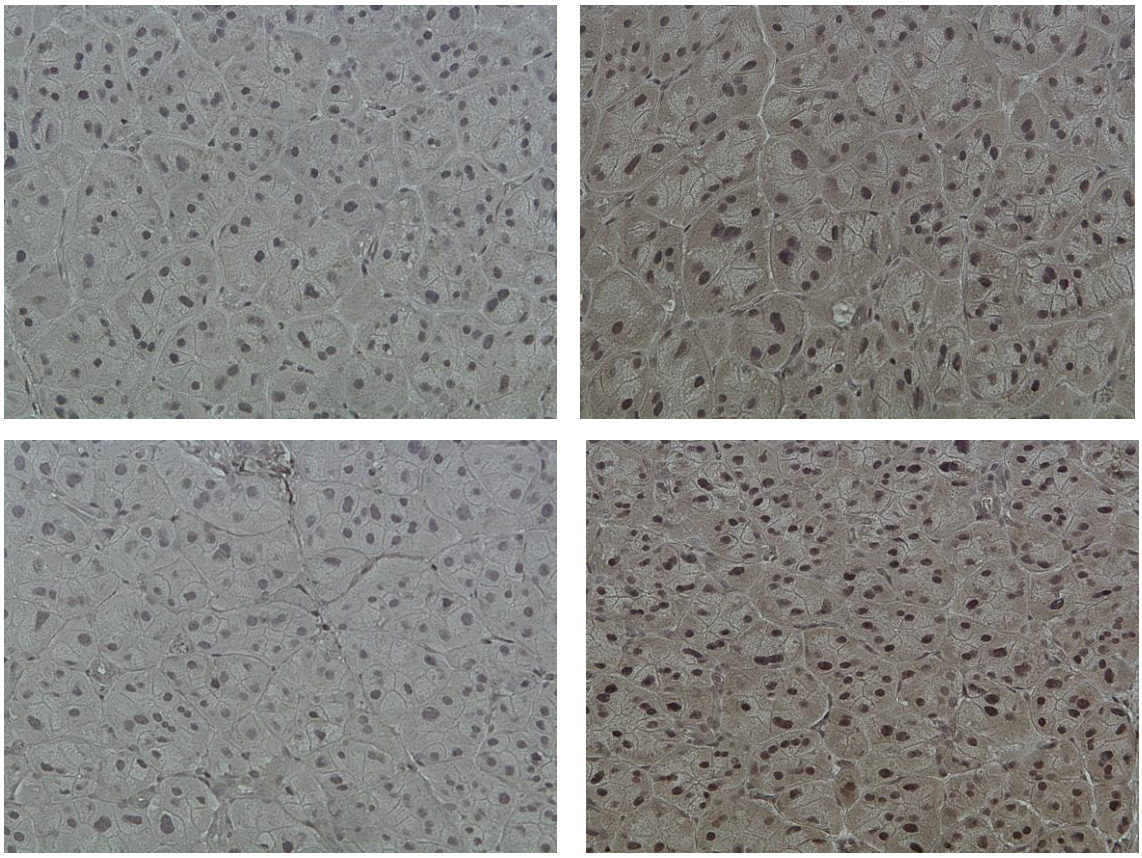

Fig. 10. Immunohistochemical labeling of the M5 muscarinic receptors in the rat lacrimal gland by immunoenzyme method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the staining for M5 receptors. All sections were counterstained with Mayer's haematoxyline.

Fig. 10. shows the results of immunohistochemical labeling of the M5 muscarinic receptors in the rat lacrimal gland. The pictures show positive staining for the M5 receptor, which is manifested by specifically brown coloured areas around the basolateral membranes of the acinar cells when compared to the negative controls. The nuclei are also of darker colour than on the controls. The LPS-treated lacrimal tissue in the lower left corner shows similar localization and intensity of staining as the healthy tissue in the upper right corner.

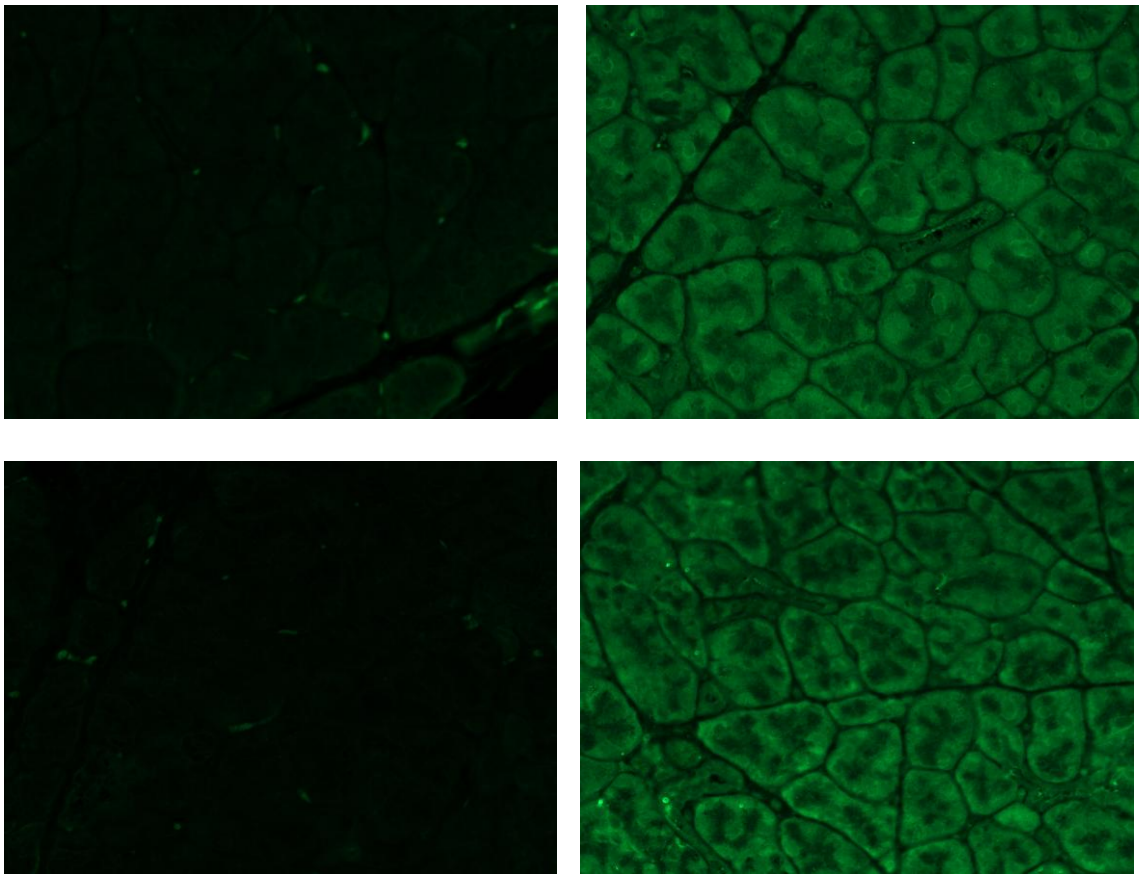

Fig. 11. Immunohistochemical labeling of the M1 muscarinic receptors in the rat lacrimal gland by immunofluorescent method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the labeling of M1 receptors.

Fig. 11. shows immunofluorescent labeling of the M1 muscarinic receptor in the rat lacrimal gland. The controls on the left side show almost no or very weak emission of light, the bright spots are just artefacts. However, the pictures on the right side, which were exposed to the primary anti-muscarinic M1 receptor antibody, show pronounced and specific emission of light, which can be observed as green colour. On the tissue we can see acini separated by thin layers of connective tissue, but the actual acinar cells cannot be distinguished. The pattern of binding of the primary antibody is similar to the pattern seen on the pictures stained by immunoenzyme method, and is situated on the basolateral membranes of the acinar cells. The LPS-treated tissue in the left lower corner shows a similar intensity of colour as the healthy tissue.

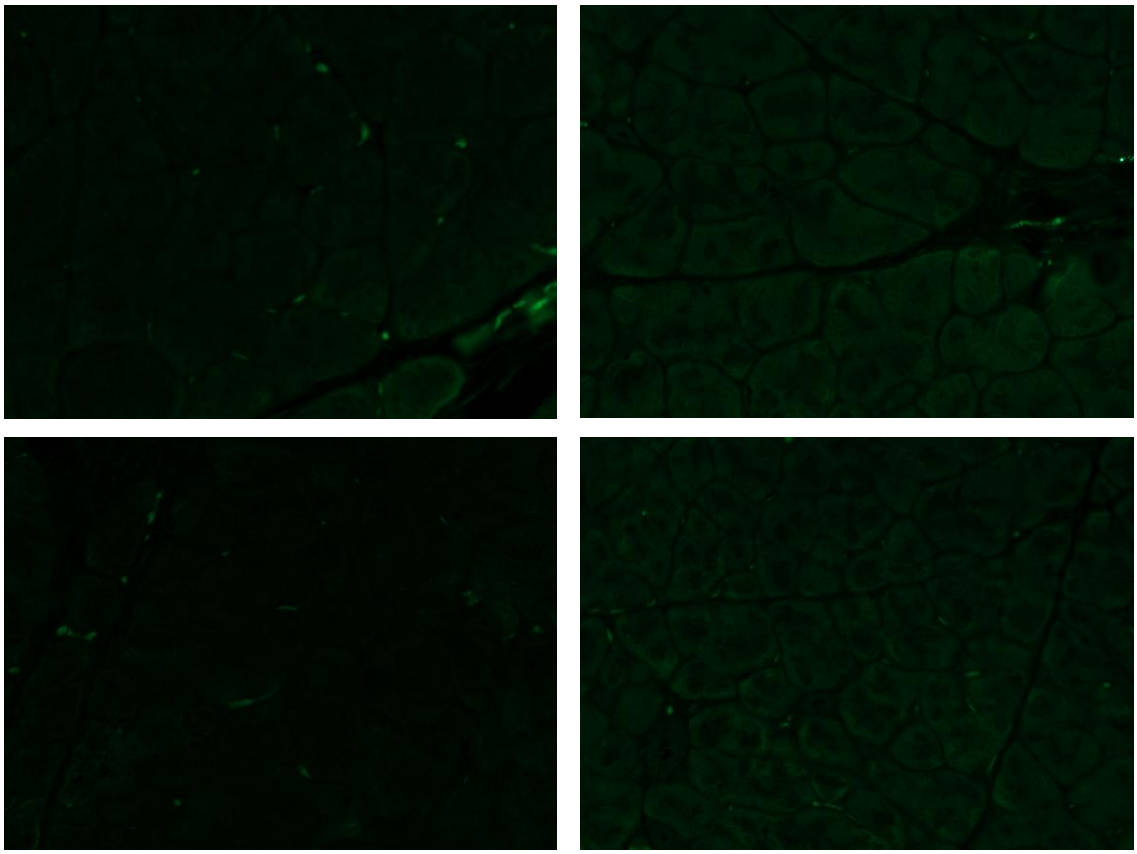

Fig. 12. Immunohistochemical labeling of the M3 muscarinic receptors in the rat lacrimal gland by immunofluorescent method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the labeling of M3 receptors.

Fig. 12. shows immunofluorescent labelling of the M3 muscarinic receptor in the rat lacrimal gland. The pictures on the right show slightly brighter green colour than the controls, and even though the emission is not very intensive, the presence of the receptors is clearly visible on the basolateral portions of the acinar cells. The intensity of the labeling is rather weak, in accordance with the intensity of staining for the M3 receptor in the immunoenzyme method. The healthy and LPS-treated tissue shows similar intensity of light emission.

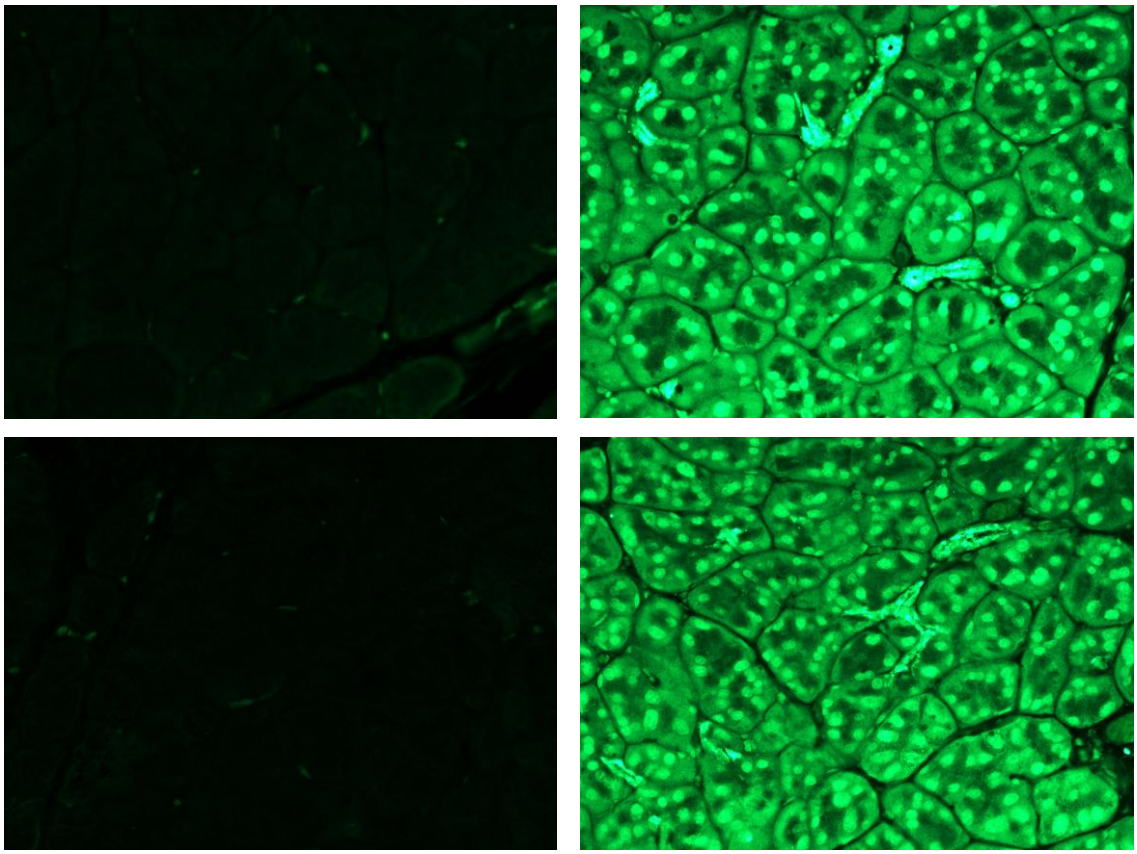

Fig. 13. Immunohistochemical labeling of the M5 muscarinic receptors in the rat lacrimal gland by immunofluorescent method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the labeling of M5 receptors.

Fig. 13. shows immunofluorescent labeling of the M5 muscarinic receptor in the rat lacrimal gland. On these pictures also several small ducts can be observed. The emission of light by the bound fluorescent antibody is very apparent in the tissues on the right side, which was incubated with the primary anti-muscarinic M5 antibody. The labelling is very obvious around the basolateral membranes of the acini and looks very much alike to the labeling pattern of the immunoenzyme method, including the bright spots resembling the nuclei. The bright green colour is apparent on both the acinar and the ductal cells. There is no significant difference between the colour intensity of the healthy and LPS-treated tissue.

5.2 Immunohistochemistry of the submandibular gland

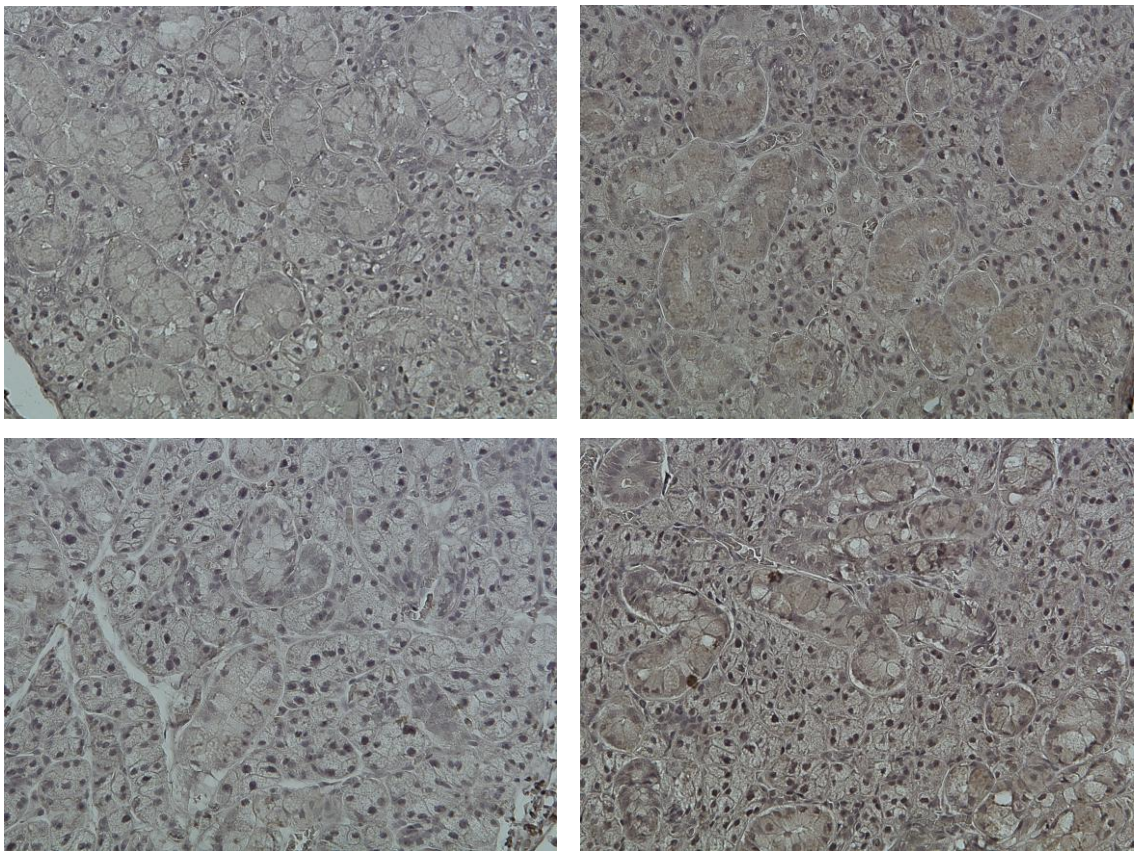

Fig. 14. Immunohistochemical labeling of the M1 muscarinic receptors in the rat submanidbular gland by immunoenzyme method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the staining for M1 receptors. All sections were counterstained with Mayer's haematoxyline.

Fig. 14. shows immunohistochemical labeling of the M1 muscarinic receptors in the rat submandibular gland. Characteristic structures of the submandibular gland can be observed on the pictures, namely the acini composed of the smaller serous cells and the acini composed of larger mucous cells . The cells are again polarized in structure, the nuclei are situated closer to the basolateral membrane, which is especially visible in the mucous cells. Specific staining for the M1 receptor occurs in both the serous and mucous cells, in both the healthy and the LPS-treated specimens. However, the staining in the mucous cells seems to be more intense.

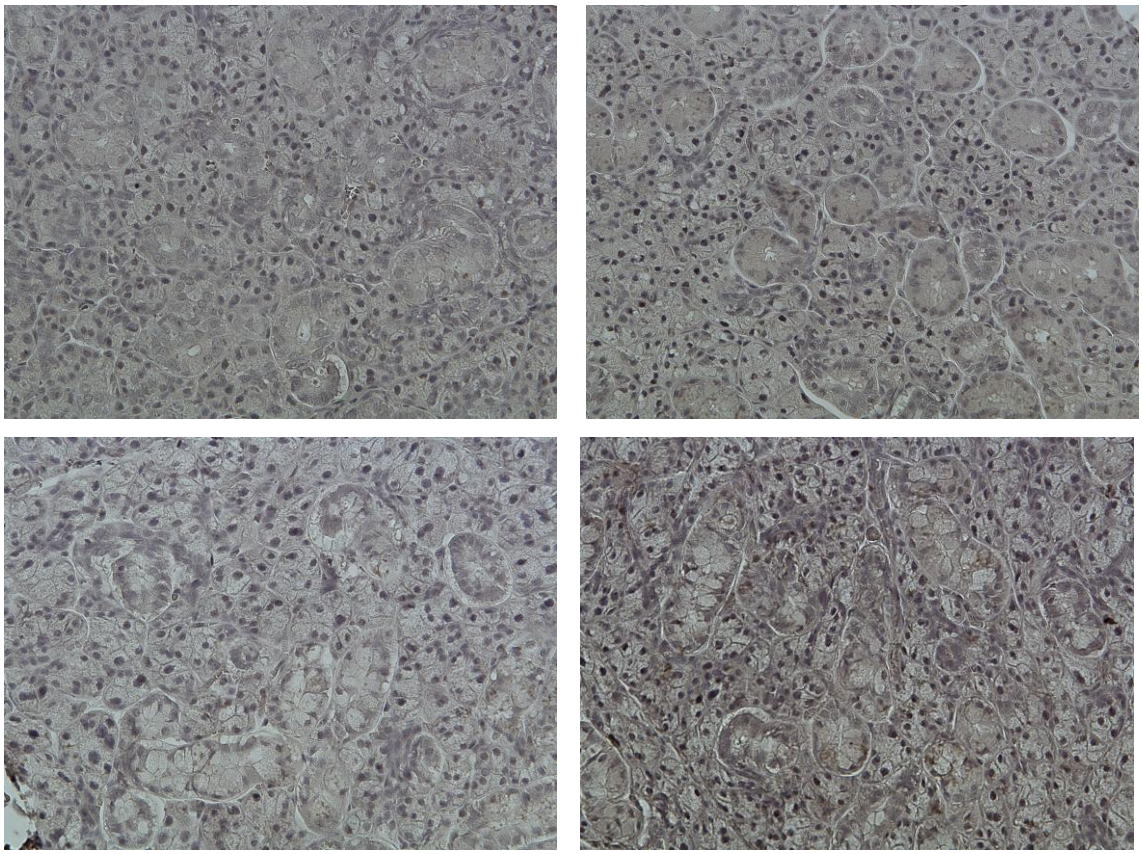

Fig. 15. Immunohistochemical labeling of the M3 muscarinic receptors in the rat submanidbular gland by immunoenzyme method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the staining for M3 receptors. All sections were counterstained with Mayer's haematoxyline.

Fig. 15. shows immunohistochemical labeling of the M3 muscarinic receptors in the rat submandibular gland. Both the healthy and the LPS-treated glandular tissue shows positive staining for M3 receptors, localized more pronouncedly in the mucous cells, but also in the serous cells. The colour intensity of the staining seems a little darker in the LPS-treated tissue.

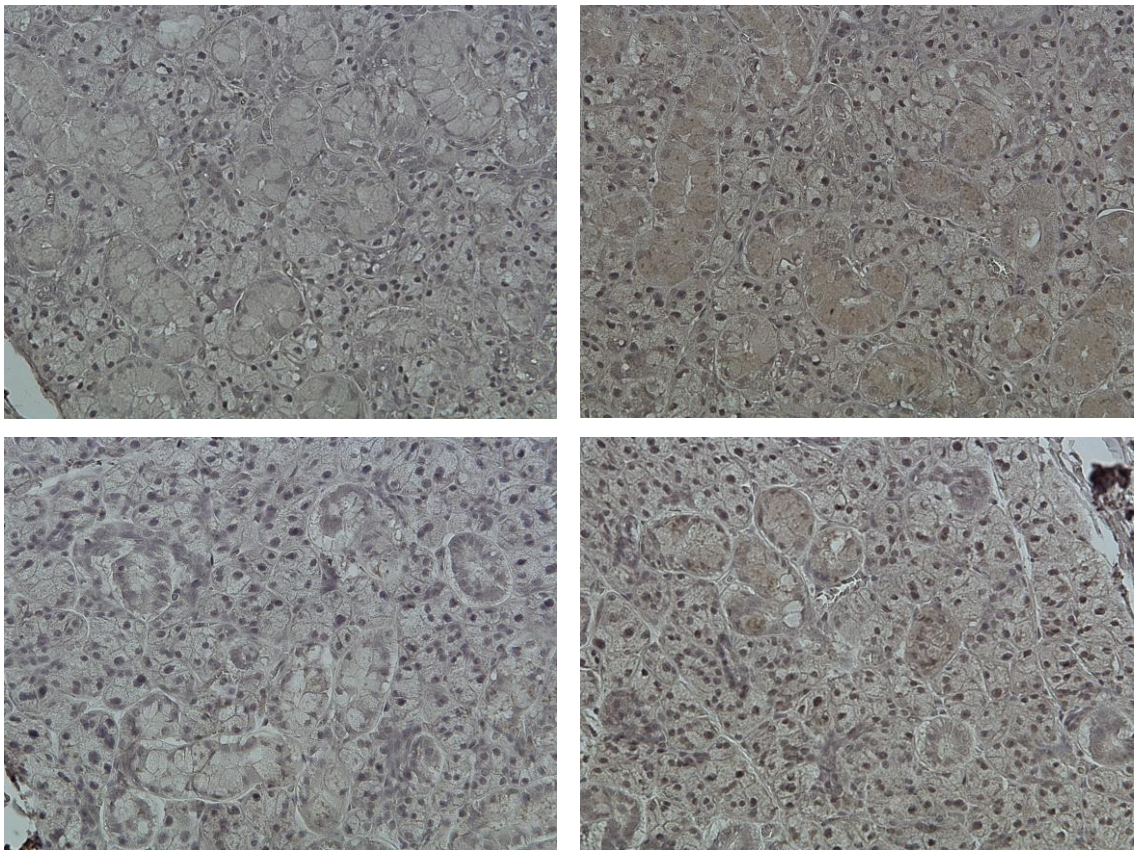

Fig. 16. Immunohistochemical labeling of the M5 muscarinic receptors in the rat submanidbular gland by immunoenzyme method. The upper row shows healthy tissue, the row below shows LPS-treated tissue. The negative controls are on the left and the right side pictures show the staining for M5 receptors. All sections were counterstained with Mayer's haematoxyline.

Fig. 16. shows immunohistochemical labeling of the M5 muscarinic receptors in the rat submandibular gland. The pictures on the left side shows controls which underwent the same procedure as the sections shown on the right side, only the primary antibody was omitted. On the positive sections, both healthy and LPS-treated, specific staining can be again observed in the mucous and serous cells, more markedly in the acini consisting of mucous cells.

5.3 Immunofluorescent labeling of human accessory labial glands

This experiment was conducted to reproduce the results from the previous report of Ryberg et al. (Ryberg, Warfvinge et al. 2008)

The ethical committee of human trials of the MAS University Hospital, Malmö, approved the examination procedures of the human tissue. This was obtained from routine biopsies for the assessment of Sjögren's syndrome. Some specimens were taken from histologically normal labial glands, while others were taken from patients with autoimmune sialadenitis compatible with Sjögren's syndrome. The glandular tissue was dissected out under local anesthesia and sent for ordinary pathological examination fixed in buffered 4% paraformaldehyde.

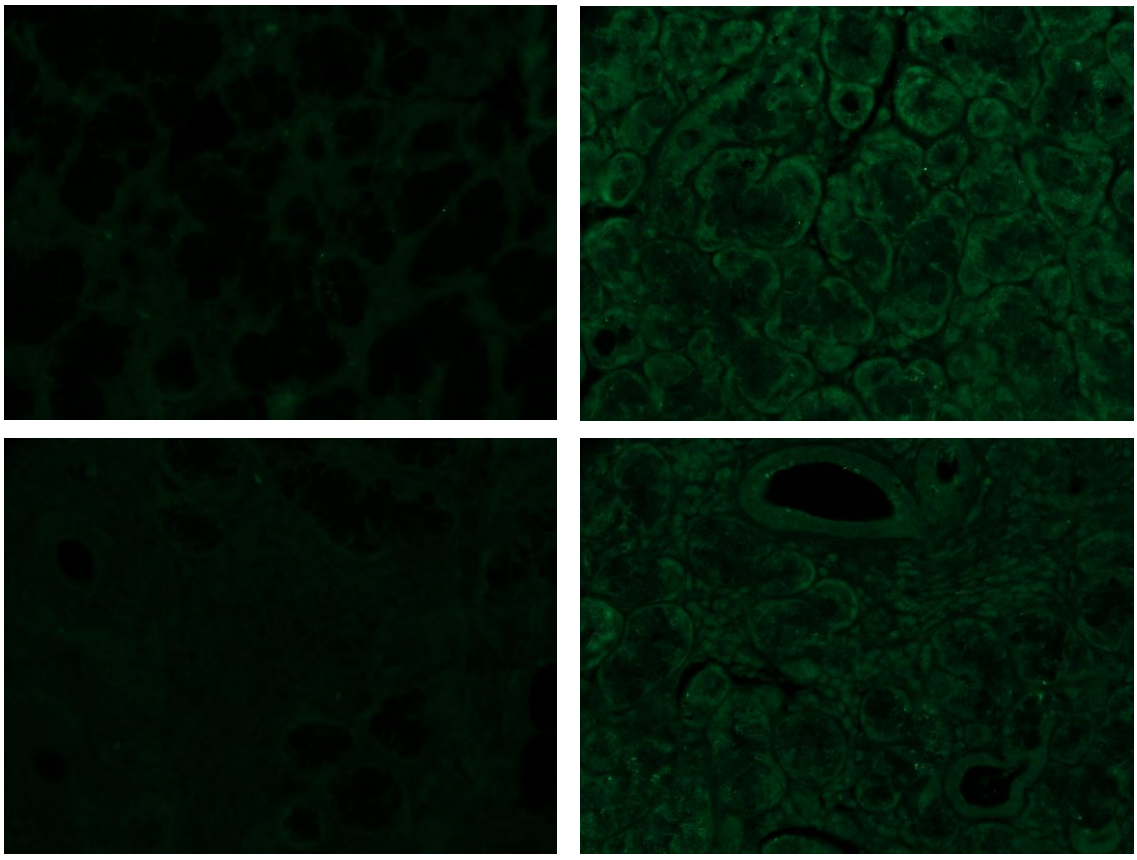

Fig. 17. Immunohistochemical labeling of the M1 muscarinic receptors in human labial gland by immunofluorescent method. The upper row shows healthy tissue, the row below shows tissue from gland with autoimmune sialadenitis. The negative controls are on the left and the right side pictures show the labeling of M1 receptors.

Fig. 17. shows immunofluorescent labeling of the M1 receptor in human healthy and sialadenitis labial glands. The labial glands consist of seromucinous acinar cells. The controls on the left side show almost no or very weak emission of light manifested by green colour. The pictures on the right side, which were exposed to the primary anti--muscarinic M1 receptor antibody, show apparent labeling of the receptors on the acinar seromucinous cells, and also on ductal cells. The sialadenitis specimen in the left lower corner shows lower expression of M1 receptors.

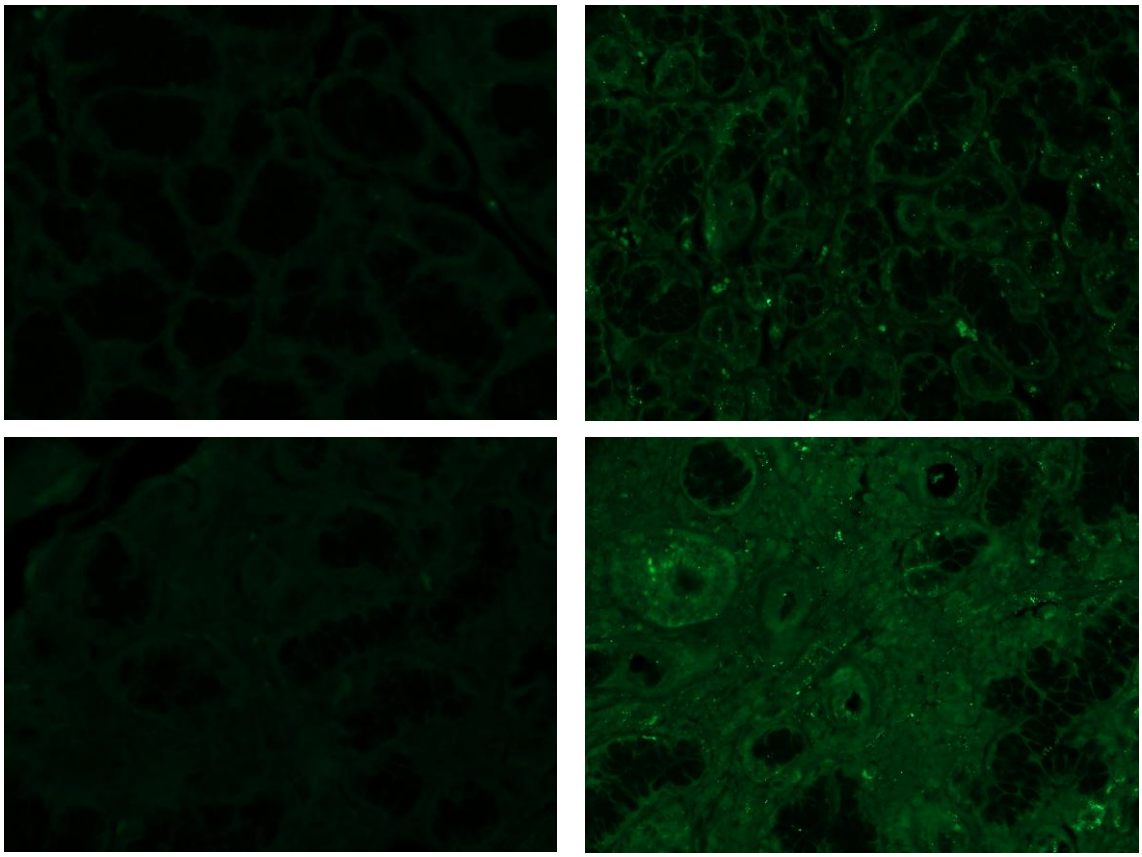

Fig. 18. Immunohistochemical labeling of the M3 muscarinic receptors in human labial gland by immunofluorescent method. The upper row shows healthy tissue, the row below shows tissue from gland with autoimmune sialadenitis. The negative controls are on the left and the right side pictures show the staining for M3 receptors.

Fig. 18. shows immunofluorescent labeling of the M3 receptor in human healthy and focal sialadenitis labial glands. Again we can observe labeling on acinar cells in the specimens incubated with the primary anti-muscarinic M3 antibody. The specimen from

patients with focal sialadenitis shows more pronounced labeling of the M3 muscarinic acetylcholine receptor.

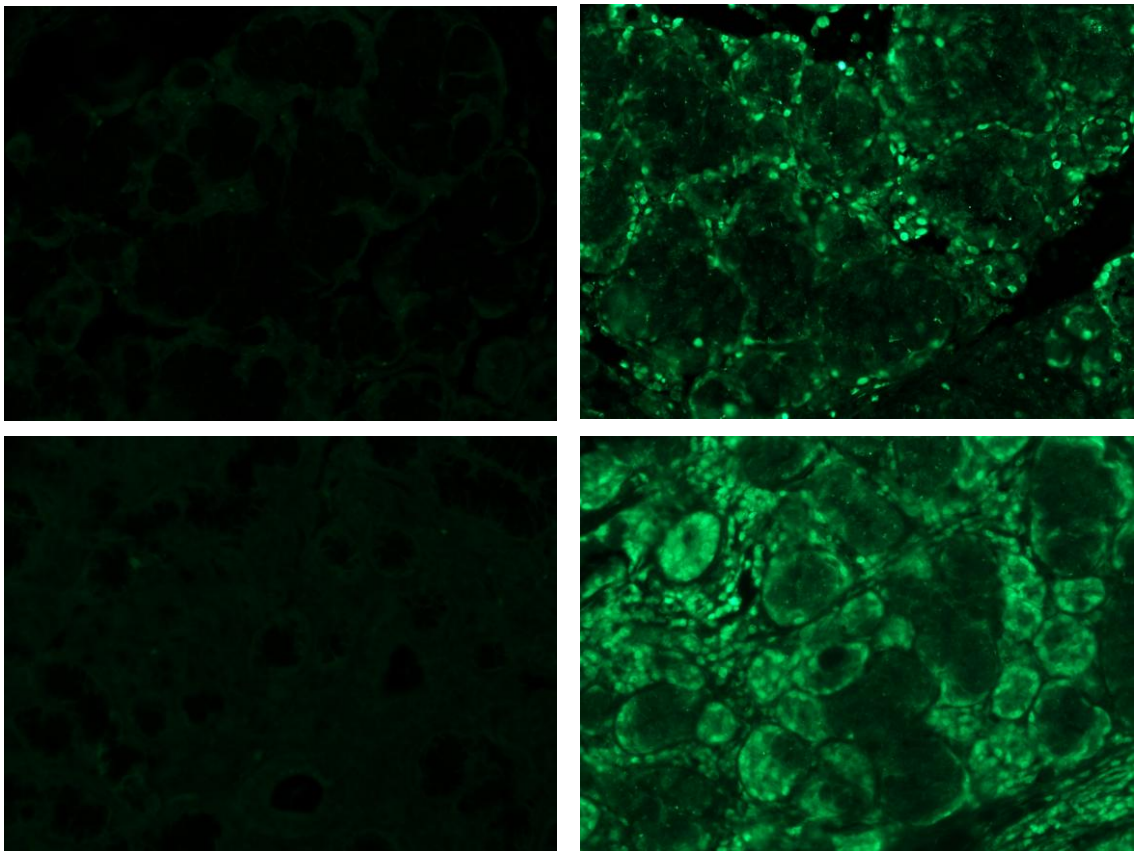

Fig. 19. Immunohistochemical labelling of the M5 muscarinic receptors in human labial gland by immunofluorescent method. The upper row shows healthy tissue, the row below shows tissue from gland with autoimmune sialadenitis. The negative controls are on the left and the right side pictures show the labeling of M5 receptors.

Fig. 19. shows immunofluorescent labeling of the muscarinic M5 receptor in human healthy and sialadenitis labial glands. The binding of the anti-muscarinic antibody is revealed by emission of light, which can be seen as green colour on the pictures. The expression of the M5 receptors in the healthy tissue is apparent mainly in and around the acini in a patchy pattern. The sialadenitis specimen shows significantly more pronounced labeling.

5.4 Summary of the immunohistochemistry results

Table 2. *Results summary of the immunohistochemistry of the lacrimal gland*

Receptor	Healthy tissue - IE method	Healthy tissue - IF method	LPS-treated tissue - IE method	LPS-treated tissue – IF method
M1	+	++	+	++
M3	+	(+)	(+)	(+)
M5	++	+++	++	+++

Parenthesis indicate weak labeling and +++ indicates very pronounced.

Table 3. *Results summary of the immunohistochemistry of the submandibular gland*

Receptor	Healthy tissue - IE method	LPS-treated tissue - IE method
M1	+	+
M3	(+)	+
M5	+	(+)

Parenthesis indicate weak labeling and ++ indicates very pronounced.

Table 4. *Results summary of the immunohistochemistry of the human labial glands*

Receptor	Healthy tissue - IF method	Focal sialadenitis tissue - IF method
M1	++	(+)
M3	(+)	++
M5	+	+++

Parenthesis indicate weak labeling and +++ indicates very pronounced.

6 Discussion

Muscarinic receptors are widely expressed all over the body's organ systems. The expression and functional significance of the muscarinic M1, M2 and M3 receptor subtypes has been thoroughly studied in most organs, while less is known about the M4 and, in particular, the M5 receptor subtypes. Muscarinic receptors have been found to carry out numerous neuronal as well as non-neuronal cholinergic functional roles. (Eglen 2006) Lately it has been suggested that the muscarinic receptors subtypes are also connected to inflammation and proliferation. (Ventura, Pennefather et al. 2002)

In the lacrimal gland, however, not that many studies have been carried out and that's why one of the aims of this thesis was to investigate the muscarinic network further. Most reviews describe a homogenous population of M3 receptors (Hodges and Dartt 2003). However, the actual experiments on which these statements are based, are not completely excluding the presence of other subtypes. In pharmacological experiments of Mauduit et al., non-selective antagonists pirenzepine and 4-DAMP were used to determine the identity of the present muscarinic receptors. (Mauduit, Jammes et al. 1993) Pirenzepine is said to block mainly M1, then M4 and then M3 and M5 muscarinic receptor. 4-DAMP is said to block M1, M3 and M5 and possibly M4 muscarinic receptors. So none of these could distinguish between M3 and M5 receptors, as so far no selective M5 antagonist has been synthesized. In the paper by Mauduit et al., also Northern blotting was carried out, but no probes were used for M4 and M5 receptors, and even the mRNA for M2 receptors in the heart could be detected only very weakly, although the presence of this subtype is generally well proved. This indicates possible problems with the sensitivity of the performed method. In the publication of Ubels et al., the M3 subtype is also stated as the only muscarinic receptor subtype present in the lacrimal gland based on cDNA microarray analysis using the whole rat embryo as a reference. (Ubels, Hoffman et al. 2006) In the paper of Nakamura et al., the M3 subtype was found to carry out cholinergic-mediated protein secretion, but again, only relatively selective antagonists were used, namely pirenzepine, gallamine and 4-DAMP, so the presence and a functional role for the M5 receptor cannot be completely excluded. (Nakamura, Tada et al. 1997)

In this thesis project, the rat lacrimal gland was investigated for the presence of muscarinic receptors using the method of immunohistochemistry, both immunoenzyme

and immunofluorescent. Anti-muscarinic M1, M3 and M5 receptor antibodies were used. The presence of the receptors was revealed by the comparison of the brown staining intensity (in the immunoenzyme method) or by the green light emission intensity (in the immunofluorescent method) of the specimens incubated with primary anti-muscarinic receptor subtype antibody to the negative controls, where the primary antibody was omitted. The results suggest the presence of all three subtypes.

As seen on Fig. 8., in the specimens incubated with anti-M1 antibody, specific brown staining can be observed in the basolateral portion of the acinar cells, which is in agreement with the histological structure of the lacrimal acini, as the acinar cells are highly polarized and the receptors are generally localized in the basolateral part. In the immunofluorescent method, the results shown in Fig. 11., the labeling pattern is the same as in the immunoenzyme method and is clearly apparent. This suggests that the M1 receptor may be present in the lacrimal gland, and as previous functional studies haven't shown any significant role in the protein secretion, it may possibly participate in other functions.

The results from the slides incubated with the anti-M3 antibody (Fig. 9. and 12.) show a weaker signal according to both methods, but still the presence of the receptors is visible. The receptors are again localized on the basolateral side of the acinar cells. As the antibodies for different muscarinic receptor subtypes have different binding properties, it's not possible to compare the extent of the receptor expression of the different receptor subtypes. That's why even though the M3 subtype shows weaker signal than the others, only the presence of the receptor can be assumed. The possible explanation for the weaker signal, even though the presence of the M3 receptor is well-proved, may be that the anti-M3 antibody has generally weaker binding potency than the antibodies for the other subtypes, as previously experienced in immunoblotting by this group (Ryberg, Warfvinge et al. 2008). Also even though the M3 receptor seems to be the most important for the secretory function of the lacrimal gland, it may not be the quantitatively most present.

The results for the M5 receptor are somewhat unexpected. The immunoenzyme method (Fig. 10.) showed intense brown staining in the basolateral portions of the acinar cells, suggesting the presence of the M5 receptor. The colour of the nuclei is also much darker then on the negative controls. The immunofluorescent method (Fig. 13.) showed a very pronounced signal in both acinar and ductal cells and moreover, in structures resembling the nuclei. In fact the labeling pattern is very similar to that of the

immunoenzyme method, pointing to a possible presence of the M5 muscarinic receptor subtype on the nuclear membrane. This was previously reported by Lind et al. (1995), who used a covalently binding muscarinic acetylcholine receptor-specific ligand to examine the expression of muscarinic receptor subtypes in the rabbit corneal epithelial and endothelial cells and in subcellular fractions of these cell types. Their results suggest that mAChR ligand-binding structures may be present on the nuclear membranes of the corneal cells, and one of these structures resembled the M5 subtype. (Lind and Cavanagh 1995) This suggestion could be very well related to the reported role of the M5 receptor in cellular growth (Kohn, Alessandro et al. 1996) and the role of muscarinic receptors in proliferation (Klapproth, Reinheimer et al. 1997), as well as to the possible anti-apoptotic potency of the M5 receptor (Budd, McDonald et al. 2003).

Acetylcholine elicits its effects influencing inflammation within different organs mainly via the α7 subunit of the nicotinic acetylcholine receptors, but muscarinic receptors have been shown to participate in inflammatory processes as well. The M5 receptor has been shown to be up-regulated in lymphocytes upon immunological stimulation (Fujii, Watanabe et al. 2003) and in the rat urinary bladder under cystitis conditions (Giglio, Ryberg et al. 2005). This leads to the next aim of the current thesis, to compare the muscarinic receptor expression in the healthy tissue and the tissue with experimentally induced inflammation. For this purpose injection of lipopolysacharide was used. The organs were removed after 24 hours and they showed apparent macroscopical signs of inflammation, such as swelling and redness.

In the lacrimal gland, no significant difference could be seen in the intensity of labeling of the M1, M3 and M5 receptors when comparing the labeled healthy tissue opposed to the control and the LPS-treated labeled tissue opposed to its own control. The LPS-treated tissue showed the same pattern of labeling for all the three muscarinic receptor subtypes, and the slightly different shade and organization of the cells on the healthy and LPS-treated tissue is probably due to slightly different angle of cutting while the tissue was processed. Similar results were obtained by both immunoenzyme and immunofluorescent method. No conclusions can be drawn about up- or down-regulation of the M1, M3 and M5 receptors under these conditions. This suggests that either there is no change in the expression of these receptor subtypes, or the inflammation was not powerful enough to trigger these changes or, most likely, longer, up to chronic inflammatory conditions are required to change the expression of these muscarinic receptors.

In the submandibular gland, the muscarinic network has been previously well described and M1, M3 and M5 receptors were all shown to be present. (Ryberg, Warfvinge et al. 2008) The results of this thesis confirm these findings. Specific staining was achieved for the M1, M3 and M5 receptors in both the serous and mucous acinar cells, although the staining seemed to be more pronounced in the mucous cells. The labeling of the M3 receptors (Fig. 15.) showed again weaker signal, as experienced in the lacrimal gland. The intensity of the staining in healthy and LPS-treated tissue was very similar, and didn't allow for drawing any conclusion about up- or down-regulation. The reason for this has been already discussed. The slightly different shade of the staining and organization of the tissue is again probably caused by the different angle of cutting while processing the specimens.

The next aim of this thesis was to investigate if there were any changes in expression of muscarinic receptor subtypes in healthy and focal adenitis human labial glands, which served as a model for Sjögren's syndrome. These experiments were conducted to confirm the previous results of Ryberg et al. (Ryberg, Warfvinge et al. 2008) The results of this thesis are in complete agreement with the above mentioned publication.

The M1 receptors have been detected on the seromucinous acinar cells of the labial glands and on the ductal cells (Fig. 17.). The specimen with focal sialadenitis showed weaker labeling of the receptors, suggesting down-regulation of this subtype during the progression of Sjögren's syndrome. As the M1 receptor is involved in the control of high-viscosity salivary secretion (Abrams, Andersson et al. 2006) and maximal salivation can be reached by simultaneous activation of both M1 and M3 receptors (Culp, Luo et al. 1996), the down-regulation can be an additive factor leading to hyposalivation.

The M3 receptor was also present on acinar cells (Fig. 18.). The up-regulation of the M3 subtype expression in the focal sialadenitis specimen was apparent when compared to the healthy tissue, which is consistent with previous reports. (Beroukas, Goodfellow et al. 2002; Ryberg, Warfvinge et al. 2008) This may be explained by the presence of autoantibodies aimed at the M3 muscarinic receptor in Sjögren's syndrome patients. The up-regulation of the M3 receptors is probably the result of their long-term blockade by these autoantibodies.

The M5 receptors could be seen on the sections in and around the acini in a patch-like pattern (Fig. 19), which may resemble the labeling pattern of the M5 receptor in the lacrimal gland (Fig. 13). However, the focal sialadenitis specimen showed much more

pronounced expression of the M5 receptor, pointing to the up-regulation of this subtype during Sjögren's syndrome. This phenomenon seems to be a common feature of chronic inflammation. The M5 receptor was reported to be coupled to activation of NO synthase (Wang, Zhu et al. 1994) and it was suggested to be involved in the regulation of production of NO in lymphocytes. (Costa, Auger et al. 1995) In the lacrimal gland, increase in NO production was proposed to play a role in lacrimal cell death in Sjögren's syndrome. (Beauregard, Brandt et al. 2003) Possible analogies could therefore be implied, but the functional significance of the M5 receptor in Sjögren's syndrome needs to be defined in further experiments.

The outcomes of this thesis show that the muscarinic receptor expression in the lacrimal gland should be investigated further, as it seems that the M3 receptor is not the sole muscarinic receptor in the lacrimal gland. The function of the other subtypes needs to be determined. Following experiments investigating the changes in muscarinic expression during inflammation should also be carried out, as these as the changes in the muscarinic network may be relevant in Sjögren's syndrome, as well as other dry eye diseases.

7 Conclusions

The present study shows:

1) The M3 receptor is probably not the exclusive muscarinic receptor subtype in the lacrimal gland, M1 and M5 receptors may also be present. The M5 receptor may even be localized intracellularly, possibly on the nuclear membrane.

2) No conclusions could be drawn about the changes in the muscarinic receptor expression in the lacrimal and submandibular glands under normal and inflammatory conditions.

3) Our model of inducing inflammation is probably not effective enough to induce changes in muscarinic receptor expression, or chronic inflammatory circumstances are necessary.

4) In human labial glands with autoimmune focal sialadenitis compatible with Sjögren's syndrome, the following changes in the expression of muscarinic receptors were detected: slight down-regulation of the M1 subtype, up-regulation of the M3 and M5 subtype.

8 Appendix

8.1 Protocol to immunohistochemistry – Immunoenzyme ABC method

Day 1

1) Incubate the sections at 60 ºC for 60 minutes.
2) Deparafination:
   2 x 30 minutes in xylene
   2 x 5 minutes in 99,5 % ethanol
   2 x 5 minutes in 95 % ethanol
   5 minutes in 85 % ethanol
   5 minutes in 70 % ethanol
3) Rinse in running water for 10 minutes.
4) Wash in TBS 2 x 5 minutes. Prepare 1 l of citrate buffer.
5) Rinse in citrate buffer for 5 minutes. Cook in citrate buffer for 6 minutes and then 3 x 5 minutes at max effect in microwave oven. Refill the evaporated citrate buffer to 0.2 l in each round to avid the section from cooking dry.
6) Cool for 30 minutes in citrate buffer.
7) Wash in TBS 2 x 5 minutes.
8) Mark the area around the section using a hydrophobic pen. The marking should stay in place during the whole experiment; if leakage occurs, it has to be improved.
9) Block non-specific protein binding with 5% goat serum in TBS for 30 min. (Depending on the size of the section the need may vary from 25 to 100 µl per section)
10) Incubate with primary antibody 100x diluted in 1 % goat serum in TBS overnight. The box for placing the sections in during the incubation should be closed, sealed by tape and filled with 200 ml of water to prevent the sections from drying. The negative controls are incubated with only 1% goat serum in TBS.

Day 2

1) Wash in TBS 2 x 5 minutes.
2) Incubate with anti-rabbit secondary antibody of the ABC kit for 30 minutes in the box under the same conditions as during the night. The solution is to be prepared according to the manual for the Santa Cruz ABC kit. (Mix 75 µl of normal goat blocking serum, 5 ml TBS and 25 µl of biotinylated secondary antibody.) All the solutions of the ABC kit should be prepared fresh, especially the peroxidase substrate.
3) Wash in TBS 2 x 5 minutes.
4) Prepare the AB reagent of the ABC kit (Mix 50 µl of reagent A-avidin, 50 µl of reagent B-biotin and 2.5 ml of TBS).
5) Incubate with the AB enzyme reagent for 30 minutes.
6) Wash in TBS 2 x 5 min.
7) Prepare the peroxidase substrate of the ABC kit (mix 1,6 ml of MiliQ water, 5 drops of substrate buffer, 1 drop of DAB chromogen and one drop of peroxidase substrate).
8) Incubate with peroxidase substrate until the section turn brown (usually 5-6 minutes).
9) Rinse carefully in running deionised water for 10 minutes.
10) Stain with haematoxyline for 5-6 minutes until the sections get intense red/purple colour.
11) Rince in 37°C running water until the sections turn blue.
12) Immerse the slides into ethanol increasing concentrations of ethanol and two times xylene, 10 minutes per bath.
13) When almost dry, put a small drop of Pertex on the top of each section and cover with a coverslip.

Solutions

Citrate buffer:

A: 0,1 M citric acid: 21,01 g $C_6H_8O_7xH_2O$ in 1000 ml distilled H_2O (dH_2O)

B: 0,1 M sodium citrate: 29,41 g $Na_3C_6H_5O_7x2H_2O$ in 1000 ml dH_2O.

At use, mix 18 ml A, 82 ml B and 900 ml dH_2O.

TBS:

A: 10xNaCl: 85 g NaCl (Sigma) in1000 ml dH_2O.

B: 10xTrizma base: 60,57 g Trizma base (tris (hydroxymethyl) aminomethan) in 1000 ml dH_2O.

At use, mix 100 ml A, 100 ml B and 800 ml dH_2O.

8.2 Protocol to immunohistochemistry – Immunofluorescent method

Day 1

The protocol for the first day is the same as for the previous method.

Day 2

1) Wash the sections 2 x 5 min in TBS.
2) Incubate, in a closed box, with fluorescent secondary antibody (1:250) in TBS containing 1 % of BSA for 1 hour (**Light sensitive)**
3) Wash the sections 2 x 5 min in TBS. Work in darkness or shadow.
4) Immerse the section for 10minutes in each bath:

    70 % ethanol

    85 % ethanol

    (2 x) 95 % ethanol

    (2 x) 99,5 % ethanol

    Work in darkness or shadow.
5) Let the sections dry in darkness. Mount with ProLong medium, just a small drop for each section and put the cover glass on it. Sections are to be stored in darkness.

9 Abbreviations

ABC – avidin-biotin complex

ADP – adenosine-5‘-diphosphate

ANS – autonomic nervous system

ATP – adenosine-5‘-triphosphate

BSA – bovine serum albumin

cAMP – cyclic adenosine-5‘-monophosphate

CGRP – calcitonine gene-related peptide

CRP – C-reactive protein

DAB – 3-3‘-diaminobenzidine

DAG – diacylglycerol

4-DAMP – 4-diphenyl-acetoxy-N-methyl-piperidine

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

GDP – guanosine-5‘-diphosphate

GTP – guanosine-5‘-triphosphate

IgE – immunoglobulin E

IHC – immunohistochemistry

1,4,5-IP_3 – 1,4,5-inositol triphosphate

IL – interleukin

LPS – lipopolysaccharide

mAchR – muscarinic acetylcholine receptor

MAPK – mitogen-activated protein kinase

NANC – non-adrenergic, non-cholinergic

NO – nitric oxide

PDGF – platelet-derived growth factor

PIP_2 – phosphatidylinositol-bisphosphate

PLC – phospholipase C

PLD – phospholipase D

PKC – protein kinase C

TNF – tumour necrosis factor

VIP – vasoactive intestinal peptide

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